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(71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors: IRVINE, Kari, R.; 2813 38th Street, N.W., Washington, DC 20007 (US). ROSENBERG, Steven, A.; 10104 Iron Gate Road, Potomac, MD 20854 (US). RESTIFO, Nicholas, P.; 1815 18th Street, N.W. #302, Washington, DC 20009 (US).

(74) Agents: FEILER, William, S. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).

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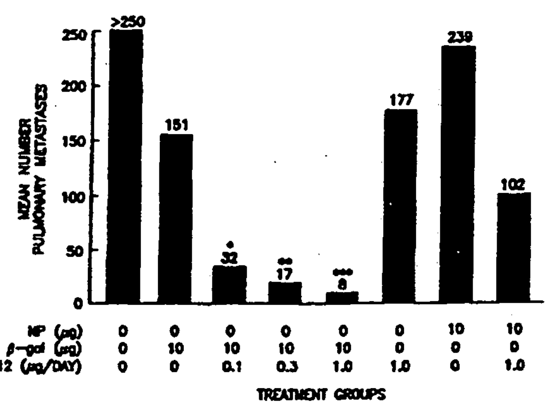

(57) Abstract

The invention provides a nucleic acid-based immunogen, preferably delivered to a recipient via a direct gene delivery system, such as a gene gun, for reducing or ameliorating pathogenic diseases, including cancers. Specifically, direct delivery immunization of the nucleic acid-based immunogens in combination with cytokine adjuvant and, if desired, one or more immunostimulatory molecules in accordance with the invention provides for the first time, prevention and treatment of a cancer or tumor. The nucleic acid immunogen in the form of plasmid DNA, for example, inoculated by particle-mediated delivery induces potent humoral and cell-mediated immune responses that result in protective immunity from tumor challenge. Active therapeutic responses in tumor bearing mammals are elicited when cytokines (for example, IL-2, IL-6, IL-7, and IL-12) are administered as adjuvant following immunization with DNA immunogens. The invention provides a novel use of the direct particle-mediated gene delivery approach of DNA immunization and of the strategy of using DNA as an immunogen in conjunction with cytokines and/or costimulatory agents for the treatment of established tumors. The invention allows several genes encoding different molecules to be coated onto each particle, and thus each bombarded cell can elaborate more than one protein, thereby aiding in enhancing and augmenting the immune response.

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ENHANCEMENT OF DNA IMMUNIZATION THROUGH THE USE OF CYTOKINES

FIELD OF THE INVENTION

The present invention relates to immunization and vaccination using nucleic acid-based immunogens for the prevention or treatment of pathogenic diseases and cancer. More particularly, plasmid DNA comprising one or more genes encoding an antigen associated with a pathogenic disease or cancer is used in accordance with the invention for immunizing or vaccinating an animal via direct delivery of the DNA to cells and tissues. The prophylactic and therapeutic effects of the nucleic acid-based vaccines of the invention are enhanced by the administration of cytokines and, optionally, costimulatory or immunomodulatory molecules.

BACKGROUND OF THE INVENTION

The induction of anti-tumor immunity, in part, involves cytolytic or cytotoxic T lymphocyte (CTL) responses (Greenberg, P.D., et al., 1981, H-2 restriction of adoptive immunotherapy of advanced tumors. J. Immunol. 126:2100; Greenberg, P.D., 1991, Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. 49:281; and Rosenberg, S.A., et al., 1988, Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. Preliminary report. N. Engl. J. Med. 319:1676). Many experimental animal studies have demonstrated that the cellular, rather than the humoral, arm of the immune response plays the major role in the elimination of murine tumors (Wunderlich, J.R. et al. Principles of tumor immunity: DeVita et al (eds) In: Biologic Therapy of Cancer, Philadelphia: J.B. Lippincott Co. 1991, pp. 3-21). Much of this evidence was derived from studies in which the adoptive transfer of T lymphocytes from immune animals was shown to transfer resistance to tumor challenge or, in some experiments, the actual elimination of established cancers. Thus, most strategies for the immunization of patients with a variety of cancers

have been directed at stimulating strong T cell immune reactions against tumor associated antigens.

Although a number of attempts at active immunization against cancer antigens has involved whole tumor cells or tumor cell fragments, it would be most desirable to immunize specifically against unique tumor antigens that distinguish malignant from normal cells. The molecular nature of the tumor associated antigens (TAAs) recognized by T lymphocytes is poorly understood. In contrast to antibodies that recognize epitopes on intact proteins, T cells recognize short peptide fragments (about 8-18 amino acids in length) that are presented on the cell surface in association with class I or class II major histocompatibility (MHC) molecules, and it is likely that tumor associated antigens are presented and recognized by T cells in this fashion.

The recent cloning of genes encoding tumor associated antigens (TAAs) recognized by CTL makes it possible to design antigen specific recombinant viral and nonviral vectors that allow for control over parameters such as the quantity and kinetics of expression, the intracellular compartment into which the TAAs are expressed and what tissues or cell types are used to express TAAs in vivo (Brichard, V., et al., 1993, The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. 178:489; Cox, A., et al., 1994, Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264:716; Kawakami, Y., et al., 1994, Cloning of the gene coding for the shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. U.S.A. 91:3515; Kawakami, Y., et al., 1994, Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc. Natl. Acad. Sci. U.S.A. 91:6458; Robbins, P.F., et al., 1995, Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24-restricted tumor-infiltrating lymphocytes. J. Immunol. 154(11):5944; Pardoll, D.M., 1995, A new look for the 1990's. Science 369:357). For example, several viruses, including recombinant vaccinia virus, fowlpox virus, and adenovirus, which encode model TAAs have been shown to express antigens within the

cytoplasm of infected cells, thereby resulting in the induction of tumor immunity (Estin, C.D., et al., 1988, Recombinant vaccinia virus against the human melanoma antigen p97 for use in immunotherapy. Proc. Natl. Acad. Sci. U.S.A. 85:1052; Kantor, J., et al., 1992, Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. J. Natl. Cancer Inst. 84:1084; and Wang, M., et al., 1995, Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J. Immunol. 154(9):4685). However, immunization with live attenuated or recombinant viruses, although potent, may also pose safety risks because of their potential infectious nature (McElrath, M.J., et al., 1994, Immune responses elicited by recombinant vaccinia-human immunodeficiency virus (HIV) envelope and HIV envelope protein: analysis of the durability of responses and effect of repeated boosting. J. Infect. Dis. 169:41). In addition, immune responses against heterologous protein may be reduced when the host has had previous exposure to the virus, such as has been observed with vaccinia virus (Graham, B.S., et al., 1993, Augmentation of human immunodeficiency virus type 1 neutralizing antibody by priming the gp160 recombinant vaccinia and boosting with rgp160 in vaccinia-naive adults. The NIAID AIDS Vaccine Clinical Trial Network. J. Infect. Dis. 167:533; Battegay, M., et al., 1993, Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. J. Immunol. 151:5408; and Cooney, E.L., et al., 1991, Safety of and immunological response to a recombinant vaccinia virus expressing HIV envelope glycoproteins. Lancet 337:567).

The identification of immunodominant peptides that represent unique tumor antigens allows new avenues for immunization against cancer. Substantial evidence exists in animal models that immunization with immunodominant viral peptides can induce viral-specific CTL that can confer protection against viral infection. Although pure peptide alone is ineffective in stimulating T cell responses, peptides emulsified in adjuvants or complexed with lipids have been shown to prime mice against challenge with fresh virus and can induce virus specific CTL that protect mice against lethal viral inocula (Kast, W.M. et al.,

1991, Proc. Nat'l Acad. Sci. U.S.A. 88:2283-2287; Deres, K. et al., 1989, Nature 342:561-564; Gao, X.M. et al., 1991, J. Immunol. 147:3268-3273; Aichele, P., 1990, J. Exp. Med. 171:1815-1820; Collins, D.S. et al., 1992, J. Immunol. 148:3336-3341). Immunization of mice against splenocytes coated with Listeria monocytogenes peptide epitopes also results in the generation of Listeria- specific CTL which can be expanded in culture. Adoptive transfer of these CTL can protect mice against lethal bacterial challenge (Harty, J.T. et al., 1992, J. Exp. Med. 175:1531-1538). Peptides representing antigenic epitopes of HIV gp120 and gp160 emulsified in complete Freund's adjuvant can also prime specific CTL responses (Takahashi, H. et al., 1988, Proc. Nat'l Acad. Sci. U.S.A. 85:3105-3109; Hart, M.K. et al., 1991, Proc. Nat'l Acad. Sci. U.S.A. 88:9448-9452).

While immunization with peptides in adjuvants or complexed with lipids gives rise to T cell responses in mice, the reactions are rarely strong enough to induce T reactive cells in primary splenocytes. The detection of sensitized lymphocytes almost invariably requires secondary in vitro stimulation.

In humans, a number of genes have been identified that encode melanoma tumor antigens recognized by tumor infiltrating lymphocytes (TIL) in the context of the HLA-A2 class I molecule (Kawakami, T. et al., 1994, Proc. Nat'l Acad. Sci. 91:3515-3519; Kawakami, Y. et al., 1994, J. Exp. Med. 180:347-352; Kawakami et al., 1994, Cancer Res. 54:3124-3126). These antigens appear to be the most clinically relevant antigens responsible for mediating tumor regression in patients with advanced melanoma, because the TIL used to identify these antigens were capable of mediating anti-tumor regression in vivo. In particular, two such antigens, which appear to be present in virtually all fresh and cultured melanomas, have been called MART-1 (Melanoma Antigen Recognized by T Cells-1) and GP-100. The genes encoding both of the peptides have been cloned and sequenced. The MART-1 gene encodes a 118 amino acid protein of 13 kD. The GP-100 gene encodes a protein identical to that recognized by monoclonal antibody HMB-45.

With the exception of melanocytes and retina, no normal tissues express these antigens and no expression of these gene products has been seen on

cancers other than melanoma. Both antigens, therefore, appear to be melanocyte lineage specific. The MART-1 antigen was expressed on nine out of nine tissue culture lines that were established from melanomas in the Surgery Branch, NCI, and on all fresh melanomas tested. Studies by others have shown that the MART-1 (also called Melan-A) antigen was expressed on 26 out of 26 fresh melanomas (Coulie, P.G. et al., 1992, J. Exp. Med. 180:35-42). The GP-100 antigen is also widely expressed in melanomas. In one study, reactivity with antibody HMB-45 (reactive with GP-100) was present on 100% of non-spindle cell type melanomas and on 62 out of 67 total melanomas (Wick, M.R. et al., 1988, J. Cutan. Pathol. 4:201-207). In another report, 32 out of 35 melanomas studied (91%) expressed GP-100 (Ordonez, N.G. et al., 1988, Am. J. Clin. Pathol. 4:385-390) and in a third study 60 out of 62 (97%) melanomas expressed GP-100 (Gown, A.M. et al., 1986, Am. J. Pathol. 123:195-203).

Of 14 separate TIL cells that were raised in the Surgery Branch, NCI from different HLA-A2 individuals, 13 out of 14 recognized MART-1 and 4 out of 14 recognized the GP-100 antigen. Because TIL cells that recognize these determinants have been shown to be capable of mediating cancer regression in vivo, it appears that these antigens are involved in cancer regression.

Another gene coding for a human tumor-specific antigen on a human melanoma was cloned by Van der Bruggen et al (1991, Science 254:1643-1647). This antigen is encoded by a gene called MAGE-1 which spans five kilobases. A 2,419 base pair coding sequence produces a predicted protein product of 26 kD. The MAGE antigen is HLA-A1 restricted and the nine amino acid fragment that represents the A1 restricted immunodominant peptide has been defined as Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr. This nine amino acid peptide is encoded by the third exon of the MAGE gene. Transfection of a 500 base pair fragment of this gene can confer recognition by a MAGE-specific CTL clone. Incubation of an EBV-infected cell line with the immunodominant peptide can confer sensitivity to lysis by a MAGE-1 specific CTL clone. MAGE-1 does not appear to be expressed in normal cells with the possible exception of testis, but is expressed on approximately half of metastatic melanomas and on about 20% of breast cancer

cells, as well as on other selected types of cancers.

In both mice and humans, the expression of the B7 gene family has been shown to be an important mechanism of anti-tumor responses. It is now becoming apparent that at least two signals are frequently required for activation of naive T-cells by antigen bearing target cells: an antigen specific signal, delivered through the T-cell receptor, and an antigen-independent or costimulatory signal leading to lymphokine products (Hellstrom, K.E. et al., 1993, Annals NY Acad. Sci. 690:225-230). Examples of two important costimulatory (also called immunostimulatory) molecules are B7-1, which is the ligand for T-cell surface antigens CD28 and CTLA4 (Schwartz, R.H., 1992, Cell 71:1065-1068; Chen, L. et al., 1992, Cell 71:1093-1102; Freeman, G.J. et al., 1989, J. Immunol. 143:2714-2722; Freeman, G.J. et al., 1991, J. Exp. Med. 174:625-631), and B7-2, an alternative ligand for CTLA4 (Freeman, G.J. et al., 1995, Science 262:813-960). To date, both murine B7-1 and B7-2 (Freeman, G.J. et al., 1991, J. Exp. Med. 174:625-631; Freeman, G.J. et al., 1995, Science 262:813-960) and human B7-1 and B7-2 (Freeman, G.J. et al., 1989, J. Immunol. 143:2714-2722; Freeman, G.J. et al., 1993, Science 262:909-911) have been described. At present, it is unclear if the costimulatory signals provided by B7-1 and B7-2 are functionally distinct or redundant mechanisms for T-cell activation (Hathcock, K.S. et al., 1994, J. Exp. Med. 180:631-640). Most murine and human tumors do not express B7-1 or B7-2, implying that even when a tumor expresses a potential rejection antigen, it is unlikely to activate anti-tumor T-cell responses (Hellstrom, K.E. et al., 1993, Annals N.Y. Acad. Sci. 690:225-230); Hellstrom, I., 1993, Annals N.Y. Acad. Sci. 690:24-31). In essence, anergy may result from only one signal being received by the T-cell (Hellstrom, K.E. et al., 1993, Annals N.Y. Acad. Sci. 690:225-230). Transfection of B7 into melanoma cells was found to induce the rejection of a murine melanoma in vivo (Townsend, S.E. et al., 1993, Science 259:368-370).

Needed in the art are safe and effective modes of immunizing individuals against pathogenic diseases and cancers. In particular, non-viral alternatives for active cancer immunotherapy would be extremely beneficial to

patients and the medical community. Direct DNA-based immunization by particle-mediated gene delivery devices is one such alternative afforded by the present invention. DNA vaccination may be accomplished by the expression of an inoculated isolated nucleic acid molecule, i.e., bacterial plasmid DNA, encoding a foreign gene of interest accompanied by other genetic sequences which enable the gene to be expressed within mammalian cells utilizing host machinery (Wolff, J.A., et al., 1990, Direct transfer into mouse muscle in vivo. Science 247:1465; Davis, H.L., et al., 1993, Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. Hum. Gene. Ther. 4:151; and Kozak, M., 1989, The scanning model for translation: an update. J. Cell Biol. 108:229).

DNA-based immunization approaches have been shown to successfully induce both humoral and cellular immunity in many antigen systems (Fynan, E., et al., 1995, DNA vaccines: protective immunizations by parental, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA 90:11478; Eisenbraum, M. D., et al., 1993, Examination of parameters affecting the elicitation of humoral immune response by particle bombardment-mediated genetic immunization. DNA and Cell. Bio. 12:791; Fuller, D. H., et al., 1995, A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune response in mice receiving a DNA-based glycoprotein 120 vaccine. AIDS Res. Hum. Retrovir. 10 (11):1433; Tang, D., et al., 1992, Genetic immunization is a simple method for eliciting an immune response. Nature 356:152: Ulmer, J. B., et al., 1993, Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745; Wang, B., et al., 1993, Gene inoculation generates immune response against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 90:4156; Sedegah, M., et al., 1995, Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proc. Natl. Acad. Sci. USA 91:9866; Pedroza Martins, L., et al., 1995, DNA vaccination against persistent viral infection. J. Virol. 69 (4):2574; and Zarozinski, C.C., et al., 1995, Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle

bombardment with DNA encoding an internal virion protein. J. Immunol. 154:4010-4017). For example, gene gun delivery of DNA encoding human growth hormone (hGH) resulted in specific antibody responses that correlated with particle delivery of the mouse epidermis, and intramuscular inoculation of plasmid DNA encoding the influenza A viral nucleoprotein induced protective humoral and cellular immunity. Protective immunity after DNA-based immunization has also been observed using gp160 and rev proteins from HIV-1, and H1 antigen from influenza, circumsporozoite protein from malaria, and nucleoprotein from LCMV. In cancer immunotherapy, plasmid constructs encoding either the full length cDNA for carcinoembryonic antigen (CEA) or HIV-1 envelope protein, gp160, have been shown to protect mice from subsequent challenge with syngeneic tumors expressing these model antigens (Conry, R.M., et al., 1994, Immune response to a carcinoembryonic antigen polynucleotide vaccine. Cancer. Res. 54:1164; Conry, R.M., et al., 1995, A carcinoembryonic antigen polynucleotide vaccine has in vivo antitumor activity. Gene Ther. 2:59; and Wang, B., et al., 1994, DNA inoculation induces protective in vivo immune responses against cellular challenge with HIV-1 antigen expressing cells. AIDS Res. Hum. Retrovir. 10:21).

In spite of the above-mentioned DNA-based approaches, therapeutic activity against established tumors using either "naked" DNA intracellular injection or gene gun immunization has not been achieved in the art. Moreover, newly presented to the art is the enhancement of the immune response and active immunotherapy following immunization, particularly against established tumors, using DNA-based vaccines in conjunction with the administration of one or more cytokines and/or costimulatory molecules, involving either co-injection of cytokine- and/or costimulatory molecule-encoding DNA by specific DNA immunization, or by systemic administration of cytokines and/or costimulatory molecules, whereby the cytokines serve as adjuvants.

SUMMARY OF THE INVENTION

The present invention provides direct delivery of immunogens or vaccines comprising isolated nucleic acid sequences for the prevention and

treatment of cancer and pathological diseases, including bacterial, parasitic, protozoan, or viral infection. The nucleic acid-based vaccines may comprise DNA, cDNA, or RNA, for example, and preferably comprise eukaryotic and mammalian vectors for expression in mammalian cells.

The invention further provides such DNA-based vaccines enhanced with the administration of cytokines to promote the active immunotherapy of cancer and treatment of infection and other pathogenic diseases. In accordance with the invention, costimulatory molecules may also be administered to enhance the immune reponse and to augment immunity via the local expression of such molecules. DNA encoding one or more cytokines and DNA encoding one or more co-stimulatory molecules may be contained within the vector comprising DNA encoding one or more tumor associated antigens or other disease-associated antigens to augment cellular and humoral immunity. Alternatively, the DNA encoding cytokines and/or DNA encoding costimulatory molecules may be contained in discrete plasmids and admixed or co-administered with the DNA encoding the tumor or disease-associated antigen(s). The invention also embraces systemic administration of one or more cytokines and/or costimulatory molecules to enhance the immune response to DNA immunization.

It is an object of the invention to provide an improved method of vaccination and immunotherapy using plasmid DNA delivered directly to cells by particle-mediated gene delivery systems, such as by means of a gene gun device, to treat and prevent cancers, metastatic cell growth, and diseases.

It is a further object of the invention to enhance nucleic acid-based immunization using one or more different cytokines in conjunction with immunization protocols designed to activate T cell immunity against bacterial or viral pathogens, as well as cancer. Nucleic acid which encodes the cytokine(s) of interest can be immunized at the same time as the DNA encoding antigen(s), e.g., tumor associated antigen(s), thereby causing local secretion of cytokines and enhancement of therapeutic immune responses. A reduction in toxicities frequently associated with such cytokines may also be provided by the invention.

Yet another object of the invention is to provide prophylactic and

therapeutic methods of cancer treatment using small amounts of DNA to induce potent and consistent cellular and humoral immune responses.

It is another object of the invention to provide a therapeutic composition and a method of preventing or treating a disease in a mammal comprising administering an effective amount of nucleic acid encoding antigen(s) of a disease-causing agent or cancer to the mammal via direct gene delivery and administering an exogenous cytokine and/or costimulatory molecule or immunomodulatory molecule, or combination thereof, for preventing or ameliorating the disease or cancer. The administration of the cytokine, costimulatory or immunomodulatory molecule can be achieved by co-injection of nucleic acid (e.g., plasmid DNA) encoding specific cytokine, immunomodulatory or costimulatory molecules with the nucleic acid encoding a disease causing agent or cancer, or by exogenous administration.

It is another object of the invention to provide a vaccine against a disease-causing agent or cancer comprising an isolated nucleic acid sequence (for example, in a plasmid) encoding one or more disease or tumor-associated antigens or immunodominant epitopes thereof, wherein the nucleic acid vaccine is directly put into cells or tissues, to prevent or reduce a cancer or a disease or infection caused by a disease-causing agent or tumor.

Additionally, it is an object of the invention to enhance the immune response resulting from nucleic acid-based vaccine in accordance with the invention by the administration of cytokines, particularly interleukins, as adjuvant to reduce an established cancer or to prevent or ameliorate pathogenic disease and cancer.

It is yet another object of the invention to enhance the immune response resulting from nucleic acid-based vaccination or immunization by the administration of one or more cytokines, particularly interleukins, and optionally one or more immunostimulatory or immunomodulating agents, chemotherapeutic drugs, antibiotics, antifungal drugs, antiviral molecules, or combinations thereof, in an amount effective for preventing or ameliorating disease, infection, or cancer.

DESCRIPTION OF THE DRAWINGS

The appended drawings of the figures are presented to further describe the invention and to assist in its understanding through clarification of its various aspects. Abbreviations used herein are as follows: CMV: cytomegalovirus; TAA: tumor associated antigen; ß-gal: beta-galactosidase; NP: nucleoprotein; CEA: carcinoembryonic antigen; rm: recombinant murine; rh: recombinant human; mAb: monoclonal antibody.

Fig. 1 demonstrates the induction of humoral immunity elicited with gene gun vaccination of the nucleic acid-based immunogen, pCMV/β-gal. BALB/c mice (three per group) were immunized two times at two week intervals in the epidermis in the abdomen; each immunization consisted of four shots of 0.25 mg of gold, thus delivering a total of either 1.0 μg of control pCMV/NP, or 0.01 μg, 0.1μg, 1.0 μg of pCMV/β-gal. Fourteen days following the boost, sera were tested by ELISA as described in Example 1 for the presence of antibodies reactive with recombinant β-gal protein. The relative concentration of antibodies reactive with β-gal was calculated from a standard curve generated using a commercially-available anti-β-gal mAb and expressed as μg/ml.

Fig. 2A-2D demonstrate that secondary in vivo T_{CD8+} cells were induced by immunization with gene gun vaccination of pCMV/β-gal. BALB/c mice were immunized one time in the epidermis; each immunization consisted of four shots of 0.25 mg of gold, thus delivering a total of 1.0 μg pCMV/NP control DNA (Fig. 2A), 0.01 μg pCMV/β-gal (Fig. 2B), 0.1 μg pCMV/β-gal (Fig. 2C), or 1.0 μg pCMV/β-gal (Fig. 2D). Fourteen days later, pooled splenocytes (two mice per group) were restimulated in vivo with 1 μg β-gal$_{876-884}$ (SEQ ID NO:1) peptide for six days and then assayed for specific lytic activity in a ^{51}Cr release assay against CT26.WT (β-gal-), CT26.CL25 (β-gal+), or CT26.WT pulsed with β-gal$_{876-884}$ (SEQ ID NO:1) peptide target cells. These experiments were repeated two times and similar results were obtained.

Fig. 3 shows that immunization with a DNA vaccine in accordance with the invention prevents the growth of intravenous tumors. Day 0, BALB/c mice were immunized one time in the epidermis, each immunization consisted of five shots of

0.25 mg of gold delivering 1.0, 0.10, or 0.01 μg of pCMV/β-gal or 1.0 μg of control DNA alone. Fourteen days later, mice (5-10 per group) were challenged, iv. with 2.5 x 10^5 CT26.CL25 (β-gal+) or CT26.WT (β-gal-) tumor cells. On day 17, lungs were harvested and tumor nodules enumerated in a blind fashion. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test, therefore error bars are not shown. The graph in Fig. 3 represents a summary of all of the data obtained from three separate experiments. In the first experiment, the control DNA utilized was pCMV/hGH; in the remaining experiments pCMV/NP was used as control DNA.

Fig. 4 shows that adoptive immunotherapy of tumor bearing mice with immune splenocytes was induced by gene gun vaccination. On day 0, CT26.WT (β-gal-, ○) and CT26.CL25 (β-gal+, ●) tumor cell lines were each injected i.v. into BALB/c mice to create lung metastases. On day three, tumor bearing mice were treated with effector splenocytes from donor mice. The donor cells were generated by prior gene gun immunization with 1 μg of either pCMV/β-gal or pCMV/NP followed 14 days later by in vitro incubation for six days with 1μg/ml of either β-$gal_{876-884}$ (SEQ ID NO:1) or $NP_{147-155}$ (SEQ ID NO:2) peptide. On day seventeen, the number of pulmonary metastases was enumerated in a coded, blind fashion. This experiment was repeated one time and similar results were obtained.

Fig. 5 demonstrates active immunotherapy of established pulmonary metastases with the pCMV/β-gal vaccine in conjunction with systemic administration of rhIL-2, rmIL-6 or rhIL-7. BALB/c mice were injected i.v. with 5 x 10^5 CT26.CL25 (β-gal+) tumor cells. On day 2 following tumor challenge, treated mice were immunized with 10 μg of pCMV/β-gal. Each mouse received 10 shots of 0.5 mg of gold with each shot delivering 1 μg of DNA. On day three, mice (5-10 mice per group) began regimens of i.p. cytokine injections as described in Example 1. On day twelve, lungs were harvested and the number of pulmonary metastases was enumerated in a coded, blind fashion. Nonparametric statistical analysis was performed using the Kruskal-Wallis test (In: Statistics, 1989, Eds., J.T. McClane and F. Dietrich II, Fourth Ed., Chapter 10.3, Collier MacMillan, p. 589). The groups with asterisks above were found to be statistically significant compared with

either cytokine alone (*: p_2=0.001; **: p_2=0.003; ***: p_2=0.0002) or with β-gal DNA alone (*: p_2=0.003; **: p_2=0.0003; ***: p_2=0.001). The Fig. 5 graph represents a summary of two experiments.

Fig. 6 demonstrates active immunotherapy of established pulmonary metastases with the pCMV/β-gal nucleic acid vaccine plus systemic administration of rmIL-12. BALB/c mice were injected i.v. with 5 x 10^5 CT26.CL25 (β-gal+) or CT26 (β-gal-) tumor cells. On day 2 following tumor challenge, treated mice were immunized with 10 μg of pCMV/β-gal or 10 μg of pCMV/NP. Each mouse received 10 shots of 0.5 mg of gold, with each shot delivering 1μg of DNA immunogen. On day three, mice began regimens of i.p. injections with rmIL-12 as described in Example 1. On day twelve, lungs was harvested and the number of pulmonary metastases was enumerated in a coded, blind fashion. Nonparametric statistical analysis was done using Kruskal-Wallis test. The groups with asterisks were found to be statistically significant compared with either cytokine alone (*: p_2=0.004; **: p_2=0.002; ***: p_2=0.002) or β-gal DNA (*: p_2=0.036; **: p_2=0.036; ***: p_2=0.012) alone. The adjuvant effects of IL-12 were observed three times, while the effect of β-gal was observed one time.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides direct nucleic acid-based immunization to induce antigen-specific cellular and humoral immune responses, as well as protective immunity for an active treatment immunotherapy against cancer and pathogenic diseases, including bacterial, parasitic, protozoan, and viral infections. In particular, DNA-based immunization offers an attractive and safe non-viral alternative for immunotherapy against cancer and other disease-causing agents and organisms.

Nucleic acid-based immunogens (also called DNA-based immunogens herein) for vaccines offer several advantages over the use of other immunizing agents, such as attenuated or recombinant viruses, for example. Purified DNA is relatively safe compared with replication-competent viruses, which may result in disseminated viremia, especially in immunocompromised

individuals. Plasmid DNA can also be easily and rapidly purified compared with the production of live viruses, which involves time consuming homologous recombination and plaque purification steps. The use of DNA vectors would also obviate the problems of anamnestic responses that can eliminate recombinant viruses more rapidly, thereby reducing immune responses against heterologous proteins expressed by viral carriers. Thus, genetic vaccines comprising isolated nucleic acid sequences represent a safe, convenient, and efficient option to other types of viral-based vaccines for the immunotherapy of cancer and pathogenic diseases.

The nucleic acid-based immunogens for prophylactic and therapeutic vaccination in accordance with the invention comprise plasmid or vector or plasmid vector DNA (these terms are used synonymously herein) comprising the isolated nucleic acid of interest, e.g., DNA, cDNA, RNA, with cDNA preferred, usually heterologous (e.g., a nucleic acid sequence (a gene) preferably double stranded, encoding a tumor-associated antigen, a nucleic acid sequence encoding an antigen of a pathogenic disease-causing microorganism or virus, and the like) accompanied by other sequences or control elements that are desired or required for proper expression of the plasmid in a cell. Plasmid vectors suitable for use in the present invention comprise at least one expression control element or promoter operationally linked to the nucleic acid sequence encoding the gene of interest and, if desired, to other nucleic acid sequences encoding cytokines, immunostimulatory or immunomodulatory molecules, or combinations thereof, as described herein. The expression control elements are inserted into the vector to control and regulate the expression of the nucleic acid sequence in a mammalian cell system, in particular. Examples of expression control elements include, but are not limited to, operator regions and promoters derived from polyoma virus, adenovirus, Rous sarcoma virus, cytomegalovirus, retroviruses, or SV40. Other promoters may include thymidine kinase (TK), phosphoglycerol kinase (PGK) or the α-actin promoters.

Alternatively, tissue-specific promoters or regulatory elements may be used, non-limiting examples of which include the following: the N-CAM

promoter (specific for brain and central nervous system); the PIT-1 promoter (pituitary specific transcription factor); the crystalline promoter (specific for protein expression in the lens of the eye); the keratin promoter (specific for protein expression in the skin); the albumin promoter (liver-specific); the alpha- or beta-globin promoters (specific for red blood cells); the Ig enhancer (specific for B lymphocytes); the T cell receptor α- or ß-promoters (specific for T cells); the insulin promoter (specific for pancreatic cells); the gastrin promoter (specific for cells of the stomach); the cardiac actin promoter (specific for heart); the tropomyosin promoter (specific for skeletal muscle); and the lactalbumin promoter and the whey acidic protein (WAP) promoter (A.C. Andres et al., 1987, *Proc. Natl. Acad. Sci. USA*, 84:1299-1303; C.W. Pittius et al., 1988, *Proc. Natl. Acad. Sci. USA*, 85:5874-5878), (specific for expression in breast tissue).

Additional preferred or required operational elements in the plasmid for immunization include, but are not limited to, enhancer sequence(s), leader sequence(s), termination codon(s), polyadenylation signals, and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in host cells. It will be understood by those skilled in the art that the correct combination of required or preferred expression control elements will depend on the host system chosen, preferably a mammalian system. It will further be understood that the expression vector should contain additional elements as necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication, splice/donor acceptor sites, such as those associated with bovine growth hormone, and selectable markers. It will be further understood by one skilled in the art that suitable mammalian cell expression vectors, or portions thereof, are routinely constructed by those having skill in the art using conventional methods and known protocols in molecular biology (Ausubel et al. 1987. In Current Protocols in Molecular Biology, John Wiley and Sons, New York, New York) or are commercially available.

As mentioned above, plasmid vectors may contain enhancer elements

to augment the amount of protein expressed intracellularly. Such additional elements may enhance or increase the levels of antigen production or persistence, as well as the quality and amounts of antigenic protein expressed in mammalian cells, in particular. Examples of regulatory response elements and enhancers that may be used in the DNA-based immunogen constructs of the invention include, but are not limited to, immunoglobulin enhancers, glucocorticoid response element (GRE), estrogen response element (ERE), metallothionein response element (MRE), heat shock response element (HSRE), and CMV intron A enhancer element (Chapman, B.S., et al., 1991, Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells, Nuc. Acids Res., 19:3979-3986).

Disease Causing Agents

The DNA-based vaccination method of the invention is effective in treating or preventing disease caused by disease causing agents or a disease state, including cancers and tumors. Each disease causing agent or disease state frequently has associated with it an antigen or immunodominant epitope on the antigen which is crucial in immune recognition by a host, and the ultimate elimination or control of the disease causing agent in a mammal, sometimes referred to in the art as a protective antigen. The mammalian immune system must come in contact with the antigen or immunodominant epitope on the antigen in order to mount a humoral and/or cellular immune response against the associated disease causing agent or cancer.

The nucleic acid-based immunogens of the invention comprises one or more isolated nucleic acid sequences encoding one or more isolated antigens or immunodominant epitopes on the antigens. The immunogen also preferably comprises one or more nucleic acid sequences encoding one or more cytokines and/or one or more immunostimulatory molecules for the purpose of enhancing or augmenting the immune response against the disease causing agent or cancer. It is to be understood that a single plasmid or nucleic acid-based immunogen may contain the above-described nucleic acid sequences encoding one or more isolated antigens or immunodominant epitopes, along with the nucleic acid sequences

encoding one or more cytokines and/or immunostimulatory molecules. Alternatively, more than one plasmid or combinations of plasmids may be used as DNA-based immunogens, for example, a plasmid comprising a nucleic acid sequence encoding one or more disease-causing antigens or an immunogenic portion thereof; a plasmid comprising a nucleic acid sequence encoding one or more cytokines; a plasmid comprising a nucleic acid sequence encoding immunostimulatory molecules, or mixtures thereof. These DNA immunogens may be delivered or administered to cells at the same time or at different times, as required or desired.

Further, and as described herein, it is to be understood that one or more cytokines and/or immunostimulatory molecules may be administered to a subject, preferably systemically, before, during, or following, preferably following, vaccination or immunization with the DNA-based immunogen to enhance the immune response to the disease causing agent. Such disease causing agents include, but are not limited to, cancers of a variety of types, pathogenic microorganisms, parasites, viruses, or mammals (and parts thereof). Mammals include, but are not limited to, non-human primates, humans, rats, mice, guinea pigs, rabbits, horses, cows, sheep, pigs, goats, and the like. Cancers of mammals which may be treated using the DNA-based immunotherapy in accordance with the invention include, but are not limited to, melanoma, carcinoma, metastases, adenocarcinoma, neuroblastoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, colon cancer, non-Hodgkins lymphoma, Hodgkins lymphoma, leukemias, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer, and the like.

The term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes or melanocyte-related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma <u>in situ</u>, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole, and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by chromosomal abnormalities, degenerative growth and

developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections, inappropriate tissue expression of a gene, alterations in expression of a gene, altered or abnormal presentation on a cell, or carcinogenic agents.

The aforementioned cancers can be assessed or treated by the compositions and methods described herein. In the case of cancer, a gene encoding an antigen associated with the cancer (e.g., a TAA) comprises the plasmid DNA used for immunization, and preferably, in conjunction with a gene encoding one or more cytokines. Genes encoding one or more immunostimulatory molecules may also be used for enhancement of the recipient's immune response. The antigen associated with the cancer may be expressed on the surface of a cancer cell or may be an internal antigen. Additionally, the internal antigen, or portions thereof, may eventually be expressed or displayed on the cell surface. In one embodiment, the antigen associated with the cancer is a tumor associated antigen (TAA) or a portion thereof. Examples of TAAs that may be used in the invention include, but are not limited to, melanoma TAAs which include, but are not limited to, MART-1 (Kawakami et al., 1994, J. Exp. Med. 180:347-352), MAGE-1, MAGE-3, GP-100, (Kawakami et al., 1994, Proc. Nat'l. Acad. Sci. U.S.A. 91:6458-6462), oncofetal antigens, CEA, TRP-1, P-15, the gp160 and rev proteins of HIV-1, the H1 antigen of influenza, the circumsporozoite protein from malaria, nucleoprotein from LCMV (Fynan, E., et al. 1995. DNA vaccines: protective immunizations by parental, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA 90:11478; Wang, B., et al. 1993. Gene inoculation generates immune response against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 90:4156; Sedegah, M., et al. 1995. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proc. Natl. Acad. Sci. USA 91:9866; Pedroza Martins, L., et al. 1995. DNA vaccination against persistent viral infection. J. Virol. 69(4):2574; Zarozinski, C.C., et al. 1995. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. J. Immunol. 154:4010-4017). and tyrosinase (Brichard et al., 1993, J. Exp. Med. 178:489), and the like.

The nucleotide sequence of the MAGE-3 gene is disclosed in Gaugler et al., 1994, J. Exp. Med. 179:921-930. MAGE-3 is expressed on many tumors of several types, such as melanoma, head and neck squamous cell carcinomas, lung carcinoma and breast carcinoma, but is not expressed in normal tissues, except for testes. The approximately 1.6 kilobase (kb) cDNA of MART-1 was cloned into a vector and the resulting plasmid has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 USA on April 14, 1994, and given ATCC Deposit Number 75738. The cloning of MART-1 is disclosed in Kawakami et al., 1994, J. Exp. Med. 180:347-352 and in U.S. Serial No. 08/231,565, filed April 22, 1994. The full length MART-1 nucleic acid sequence can be isolated from the pCRII plasmid by digestion with HindII and XhoI restriction enzymes. This 1.6kb nucleic acid sequence, or portions thereof, can be used in the DNA immunogens as described herein along with a cytokine-encoding gene or genes and/or with an immunostimulatory molecule-encoding gene or genes.

In an embodiment of the invention, the TAAs are CA-19-A (pancreatic cancer), CA-125 (ovarian cancer), PSA (prostate cancer), erb-2 (breast cancer), CA-171A and the like (Boon et al., 1994, Ann. Rev. Immunol. 12:337). However, the present invention is not intended to be limited to the genes encoding the above listed TAAs. Other TAAs, the nucleic acid of which, may be identified, isolated and cloned by methods known in the art, such as those disclosed in U.S. Patent No. 4,514,506.

As mentioned above, genes encoding an antigen of a disease causing agent in which the agent is a pathogenic microorganism in mammals may include viruses such as HIV (e.g., gp120, p17, gp160 antigens), influenza (NP, HA and H1 antigens), herpes simplex (HSVdD antigen), human papilloma virus, equine encephalitis virus, hepatitis (e.g., Hepatitis B Surface Antigen or HBSAg; HAV; HCV; HEV) feline leukemia virus, canine distemper, rabies virus, and the like. Pathogenic bacteria include, but are not limited to, Chlamydia, Mycobacteria, Legioniella, and the like. Pathogenic protozoans include, but are not limited to, malaria, Babesia, Schistosoma, Toxiplasma, Toxocara canis, and the like.

Pathogenic yeast include Aspergillus, invasive Candida, and the like. In a preferred embodiment, the pathogenic microorganism is an intracellular organism that infects cells.

Immunostimulatory Molecules: Cytokines and Costimulatory/Accessory Molecules

As described, one aspect of the invention includes a nucleic acid-based immunogen comprising a gene or genes encoding one or more costimulatory/accessory molecule(s) and/or a gene or genes encoding one or more cytokine(s) in combination with a gene encoding an antigen, or an immunogenic portion thereof, from a disease causing agent for immunization by direct DNA delivery to cells, preferably by particle-mediated introduction into cells and tissue, most preferably by means of a gene gun device, and the like. Examples of costimulatory or immunostimulatory molecules include, but are not limited to, B7-1, B7-2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72 and the like. Examples of cytokines encompassed by the present invention include, but are not limited to, IL-1, IL-2, and IL-3 through IL-9, IL-10, IL-11, IL-12, and IL-13 through IL-15, G-CSF, M-CSF, GM-CSF, TNFα, IFNα, IFNγ, RANTES (Regulated upon Activation, Normal T Expressed and presumably Secrected cytokines, Promega, G5661), and the like. Preferred cytokines include IL-2, IL-6, IL-7, and IL-12. Examples of chemokines that may also be used in the present invention include, but are not limited to, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MBSA, MIP-1α, MIP-1B, and the like.

Costimulatory molecules of the B7 family (namely B7.1, B7.2, and possibly B7.3) represent a more recently discovered, but important group of cell regulatory or immunostimulatory or accessory molecules. B7.1 and B7.2 are both members of the Ig gene superfamily. These molecules are present on macrophages, dendritic cells, and monocytes, i.e., antigen presenting cells (APCs). If a lymphocyte encounters an antigen alone, without costimulation by B7.1, it will respond with either anergy or apoptosis (programmed cell death); if the costimulatory signal is provided, it will respond with clonal expansion against the target antigen. In general, no significant amplification of the immune response against a given antigen occurs without costimulation (June et al., 1994,

Immunology Today 15:321-331; Chen et al., 1994, Immunology Today 14:483-486; Townsend et al. Science 259:368-370. Freeman et al. (1989, J. Immunol. 143:2714-2722) have reported the cloning and sequencing of the B7.1 gene. Azuma et al. (1993, Nature 366:76-79) have reported the cloning and sequencing of the B7.2 gene.

In one embodiment the B7.1 gene is present in a plasmid immunogen comprising the gene encoding the ß-gal TAA under the control of the CMV promoter. The construct for B7.2 and B7.1/B7.2 in conjunction with a tumor antigen is prepared in the same fashion as that for B7.1. In another embodiment the gene encoding a cytokine, e.g., IL-12, is also present in the construct with the gene encoding ß-gal and the gene encoding B7. In another embodiment, discrete plasmids containing the gene encoding ß-gal, the gene encoding IL-12, and the gene encoding B7 are used in admixture as immunogen and delivered to the epidermis in the gene gun particle-mediated delivery system. In another embodiment, the gene encoding the TAA (e.g., ß-gal) is delivered as the DNA immunogen by a hand-held gene gun device and cytokine (e.g., IL-12) is provided as adjuvant thereafter by systemic intraperitoneal administration.

The present invention also encompasses methods of treatment or prevention of a disease caused by the disease causing agents disclosed herein. In the method of treatment, direct gene delivery, e.g., gene-gun administration, of the DNA immunogen of the invention may be either for prophylactic or therapeutic purposes. When provided prophylactically, the immunogen of the invention is provided in advance of any disease symptom, as a prophylactic vaccination. The prophylactic administration of the nucleic acid-based immunogen serves to prevent or ameliorate any subsequent infection or disease, including tumor growth. When provided therapeutically, the nucleic acid-based immunogen may be provided at the time of or some time after (preferably shortly after, e.g., about one to five days, preferably two days after) the onset of a symptom of infection or disease, or the determination or detection of a tumor. Thus, the present invention may be provided either prior to the anticipated exposure to a disease-causing agent or after the initiation of the infection or disease or cancer or establishment of a tumor.

The genetic definition of tumor-associated antigens allows for the development of targeted antigen-associated vaccines for cancer therapy. A plasmid vaccine comprising one or more tumor antigen-encoding genes in combination with a cytokine and/or immunostimulatory molecule is a powerful system to elicit a specific immune response in terms of prevention in patient with an increased risk of cancer development (preventive immunization), prevention of disease recurrence after primary surgery (anti-metastatic vaccination), or as a tool to expand the number of CTL in vivo, thus improving the effectiveness in the eradication of diffuse tumors or infection (treatment or therapy of established disease). Moreover, the nucleic acid-based immunogens of the present invention can elicit an immune response in patients that is enhanced ex vivo prior to being transferred back to the tumor bearer (i.e., adoptive immunotherapy).

The term "unit dose" as it pertains to the inoculum refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined quantity of plasmid or nucleic acid immunogen calculated to produce the desired immunogenic effect in association with the required diluent, carrier, or excipient. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are dependent upon the unique characteristics of the nucleic acid-based immunogen and the particular immunologic effect(s) to be achieved. The inoculum is typically prepared as DNA precipitated onto gold particles as described herein, or in solution in a tolerable (acceptable) diluent such as saline, phosphate-buffered saline or other physiologically tolerable diluent, and the like, to form an aqueous pharmaceutical composition or excipient.

The route of inoculation is preferably epidermal, although intramuscular delivery may also be used. It will be appreciated that the intramuscular route is preferred for direct immunization. Other routes of immunization include, but are not necessarily limited to, subcutaneous (s.c.), intradermal (i.d.), intrasternal, intracranial, parenteral, and the like, to result in eliciting a protective or therapeutic response against the disease causing agent. The dose is administered at least once. Immunizations employing a direct gene delivery system, such as the gene gun, may be shot into sereral tissue types, including skin,

liver, spleen, and muscle, for example. In humans, as will be appreciated by the skilled practitioner, shots may be delivered in the abdomen, and also in the back, limbs (e.g., upper arm or leg (thigh)), buttocks, or the cranium, as necessary or required. Subsequent boosting doses may be administered as indicated. In providing a mammal, preferably a human, with the nucleic acid-based immunogen of the present invention, the dosage of administered immunogen will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, disease progression, tumor burden, and the like.

In general, it is desirable to provide the recipient mammal with a DNA immunogen in the range of from about 0.001 to about 100 μg of DNA, and more preferably from about 0.01 to about 50 μg, although a lower or higher dose may be administered depending upon the size and body mass of the recipient mammal. Each direct delivery shot from a gene gun delivers 1 μg of DNA; therefore, multiple immunizing shots will be given to a recipient. For example, to immunize a mouse with about 0.01 to 10 μg DNA, the animal receives from about 1 to 10 shots. For humans and non-human primates, about 5 to 25 μg of DNA is a preferred range for immunization, and more preferably about 10 to 20 μg. Accordingly, for the latter more preferred quantity of DNA, an individual will receive about 10 to 20 shots. Immunizations may be given about 2 to 25 times (average for humans, about 10-20 times; average for mice about 2-3 times) during a course of treatment, although the number of times may be modified as required by the dosage to be administered and as determined by treatment protocol(s). For administration as an adjuvant, a cytokine may be given two to five times, preferably three times, per day. The amount of cytokine administered each time may vary, depending upon the cytokine used; however, the amount and parameters of administration for a given cytokine can be routinely determined by the skilled practitioner. For example, a typical high dose of IL-2 (e.g., rhIL-2) constitutes about 120,000 cetus units (cU)/kg administered three times per day, while a low dose of IL-2 constitutes about 12,000 cU/kg administered three times per day.

The nucleic acid-based immunogen can be introduced into a mammal

either prior to any evidence of cancer, such as melanoma, or to mediate regression of the disease in a mammal afflicted with a cancer, such as melanoma or genetically inherited cancers. In a most preferred embodiment, a hand-held helium powered gene gun device was used to achieve the intracellular delivery of DNA-coated gold particles to the epidermis. Following particle-mediated delivery, the DNA redissolves in the aqueous environment of the cytoplasm or nucleus of cells and is then available for expression. In a most preferred embodiment, epidermal gene gun immunization of DNA appeared to be most efficient at eliciting humoral and cellular immune responses in the recipient.

After immunization, the efficacy of the vaccine can be assessed by monitoring the production of antibodies or immune cells that recognize the antigen, as assessed by specific lytic activity or specific cytokine production or by tumor regression. Those having skill in the art are familiar with the conventional methods used to assess the aforementioned parameters. If the mammal to be immunized is already afflicted with cancer or metastatic cancer, the vaccine can be administered in conjunction with other therapeutic treatments.

In one method of treatment, autologous cytotoxic lymphocytes or tumor infiltrating lymphocytes may be removed from the patient with cancer as disclosed in U.S. Patent No. 5,126,132 and U.S. Patent No. 4,690,915. The lymphocytes are grown in culture and antigen specific lymphocytes are expanded by culturing with antigen or antigen bearing cells or by delivering the DNA constructs of the invention to the cells. The antigen specific lymphocytes are then reinfused back into the patient.

The present invention also encompasses combination therapy. By combination therapy is meant that the DNA-based immunogen containing one or more genes encoding one or more antigens associated with one or more disease-causing agents along with DNA encoding cytokine, and optionally, if desired, one or more genes encoding one or more immunostimulatory molecules is/are administered to the patient in combination with other exogenous immunomodulators or immunostimulatory molecules, chemotherapeutic drugs, antibiotics, antifungal drugs, antiviral drugs, and the like, alone or in combination. In one embodiment,

the combination therapy includes exogenous cytokines such as IL-2, IL-6, IL-7, and IL-12. In another embodiment, the combination therapy includes other exogenously added agents in addition to IL-2, IL-6, IL-7 or IL-12, or combinations thereof, such as cyclophosphamide, and cisplatin, gancyclovir, amphotericin B and the like.

Another aspect of the invention is an antibody or antibodies elicited by immunization with the DNA-based immunogen the present invention. The antibody has specificity for and reacts with or binds to the antigen or epitope thereof, of interest. In this embodiment of the invention, the antibodies are monoclonal or polyclonal in origin. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of immunoglobulin molecules that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'); F(ab')$_2$ and F(v). Polyclonal or monoclonal antibodies may be produced by methods conventionally known in the art. (Kohler and Milstein, 1975, Nature 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.), 1985, "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in E. coli is the subject of the PCT patent applications: publication numbers WO 901443 and WO 9014424, and in Huse et al., 1989, Science 246:1275-1281.

In one embodiment, the antibodies are used in immunoassays to detect the novel antigen(s) of interest in biological samples. In another embodiment, MART-1 antibodies generated by immunization with the DNA encoding MART-1 and the administration of cytokines, and, optionally, costimulatory molecules, are used to assess the presence of the MART-1 antigen from a tissue biopsy of a mammal afflicted with melanoma, using immunocytochemistry. Such assessment of the delineation of the MART-1 antigen in a diseased tissue can be used to prognose the progression of the disease or

cancer in a mammal afflicted with the disease or the efficacy of immunotherapy. Conventional methods for immunohistochemistry are described in Harlow and Lane (eds). 1988. In: Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York; and Ausbel et al. (eds). 1987. In: "Current Protocols In Molecular Biology", John Wiley and Sons, New York, New York. Additionally, the antibodies themselves are used for immunotherapy and in adoptive immunotherapy.

In providing the antibodies or antigen binding fragments to a recipient mammal, preferably a human, the dosage of administered antibodies or antigen binding fragments will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical condition and the like. In general, it is desirable to provide the recipient with a dosage of antibodies or antigen-binding fragments which is in the range of from about 1 mg/Kg to about 10 mg/Kg of body weight of the mammal, although a lower or a higher dose may be administered. The antibodies or antigen-binding fragments of the present invention are intended to be provided to the recipient subject in an amount sufficient to prevent, lessen, or attenuate the severity, extent, or duration of the disease or infection.

To enhance the function of DNA immunization for active immunotherapy, one or more cytokines is used as adjuvant. The cytokine(s) may be administered as DNA which is co-injected with DNA encoding the tumor or disease-associated antigen, or as an exogenous agent serving as adjuvant. In this way, cytokines involved in the activation and expansion of lymphocyte populations may improve the therapeutic effects of the DNA-based immunization.

In another aspect of the invention, immunizations were performed using a nucleic acid-based immunogen comprising an isolated nucleic acid sequence encoding a disease or cancer-associated antigen, e.g., a tumor associated antigen, using direct delivery of the nucleic acid to cells and tissues. The preferred mode of delivery of the nucleic acid-based immunogen is directly into cells and tissues using a mechanical delivery device such as a gene gun for the treatment of an established tumor or disease-causing agent or to prevent the establishment of a

tumor or disease-causing agent. In particular, DNA-based immunization was successfully used to prime T lymphocytes reactive with tumor associated antigen prior to ex vivo expansion and adoptive immunotherapy.

In a particular aspect, a model system as described and exemplified hereinbelow was employed to assess the efficacy of DNA-based immunization as a potential cancer treatment strategy. The model murine tumor was CT26, which expresses the tumor associated antigen (TAA), β-galactosidase (β-gal); the β-gal-expressing tumor is designated CT26.CL25 (see Example 1). The nucleic acid-based immunogen, comprising a plasmid expressing β-gal (i.e., pCMV/β-gal), was administered by particle-mediated gene delivery to the epidermis using a hand-held, helium driven "gene gun" and induced β-gal-specific antibody and lytic responses. Immunization with this construct prevented the growth of pulmonary metastatic tumor. In addition, the adoptive transfer of splenocytes generated by in vivo immunization of pCMV/β-gal and cultured in vitro with the β-gal$_{876-884}$ (SEQ ID NO: 1) immunodominant peptide reduced the number of established pulmonary nodules in recipient mammals. To enhance the function of DNA immunization for active immunotherapy, a panel of cytokines was added as adjuvants following DNA administration. The reduction in the number of established metastases was particularly significant when IL-2, IL6, IL-7 or IL-12 were given after DNA inoculation, with IL-12 as an adjuvant having a most profound effect in the reduction of tumor burden. Thus, the invention embraces both the immunization of DNA immunogens directly delivered intracellularly, for example, by particle-mediated gene gun administration and the administration of cytokines for the activation and expansion of lymphocyte populations to improve the therapeutic effects of DNA immunization. Given the ease with which the plasmid DNA can be prepared to high purity for safe use in humans with infectious and pathogenic diseases and cancers, DNA immunization administered together with cytokine adjuvant, and optionally, costimulatory molecules, offers an attractive alternative to other types of vaccines, such as recombinant or attenuated viral vaccine preparations.

The invention further provides the novel use of gene gun

immunization of nucleic acid-based immunogens for the prevention and treatment of cancer and pathogenic disease and/or infection. A preferred aspect is immunization by gene gun delivery of plasmid DNA encoding tumor associated antigen for the prevention and treatment of cancers and established tumors. Plasmid DNA delivered by particle-mediated delivery induced potent humoral and CTL lytic immune responses that likely afforded a protective immunity from tumor challenge (see Figs. 1 and 2A-2D). Other modes of DNA delivery, such as particle bombardment or direct intradermal or intramuscular inoculation of DNA, may also be suitable for use in the invention; however, the gene gun method offers potent and consistent antibody and CTL responses to antigen(s) using small quantities of nucleic acid as immunogen (e.g., as little as 0.1 μg to 0.01 μg of DNA). For modes of DNA immunogen delivery other than via a gene gun, for example, intramuscular injection, it will be appreciated that larger quantities of DNA may be required (e.g., at least 50 μg or more) and that muscle regeneration agents, such as bupivicaine, may be needed to facilitate the uptake and transcription of the introduced DNA and thus improve the immune responses.

With the gene gun particle delivery technique, several genes encoding different molecules can be coated onto each gold bead, thus each bombarded cell can potentially elaborate more than one protein. Thus, the invention also encompasses beads that are coated with DNA encoding immune enhancing and regulating molecules, such as cytokines and costimulatory molecules, for immunomodulating, augmenting and improving anti-tumor and anti-disease causing antigen immune effects.

Those having skill in the art will appreciate that β-gal, which is foreign to the immune system of recipient animals, is recognized as "non-self", and the responses elicited by the exemplary pCMV/β-gal constructs may be somewhat different from the responses induced by a "self" antigen". However, a number of non-self antigens do bear similarities to some potential tumor antigens such as viral antigens expressed in virally-induced tumors, mutated tumor suppressor genes (Hollstein, M., et al., 1991, p53 mutations in human cancers. Science 253:49), fusion proteins resulting from translocations (Gribben, J.G., et al., 1992, Bone

marrows of non-Hodgkin's lymphoma patients with a bcl-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. Blood 80:1083), frame-shifts, and loss of stop codons. As demonstrated herein, DNA encoding the foreign ß-gal antigen administered via the gene gun technique in conjunction with the effects of exogenously administered cytokines stimulated potent immune responses and protection in animals in vivo.

In another of its aspects, the invention provides not only potent humoral and cellular immune responses, but also therapeutic responses such that established tumors were treated in active immunotherapy, as demonstrated by the observed impact on tumor burden in a tumor bearing animal model system. This aspect of the invention was achieved by the use of one or more cytokines (particularly the interleukins, such as recombinant human interleukin-2 (rhIL-2), recombinant murine interleukin-6 (rmIL-6), recombinant human interleukin-7 (rhIL-7), and recombinant murine interleukin-12 (rmIL-12), administered following DNA administration. It will be appreciated that such cytokines and the DNA encoding the cytokines are commercially available. Examples of other immunstimulatory molecules suitable for combined use in the invention include, but are not limited to, TNF-α, IFN-γ, G-CSF, GM-CSF, and IL-1 to IL-15.

It is an beneficial advantage of the invention that active immunotherapeutic treatment was elicited when cytokines were utilized as adjuvants to DNA immunization. When a panel of cytokines was tested, IL-2, IL-6, IL-7 and IL-12 were optimally able to enhance the therapeutic responses, with IL-12 having a most profound adjuvant effect. IL-2, IL-7 and IL-12 have all been reported to stimulate or activate T cell populations as well as natural killer (NK) cells. IL-12 also functions to regulate immune responses by directing a Th2 to a Th1 phenotype (Hsieh, C.S., et al., 1993, Development of Th1 CD4+ T cells through IL-12 produced Listeria-induced macrophages. Science 260:547; Sypek, J.P., et al., 1993, Resolution of cutaneous Leishmaniasis: Interleukin 12 initiates a protective T helper Type 1 immune response. J. Exp. Med. 177:1797). IL-6, known as B cell stimulatory factor-2, is a pleiotropic cytokine that can enhance

CTL function, NK activity, LAK and TIL activity (Mulé, J.J., et al. 1992. Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol. 148:2622; Takai, Y., et al., 1988, B-cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. J. Immunol. 140:508). In addition, the above-mentioned cytokines have been reported to have anti-tumor effects when administered as single agent therapy. However, the doses of the cytokines used in the present invention had little or no effect on the growth of CT26.CL25 cells. Some cytokines, such as GM-CSF, IFN-γ, and IL-4, when adminstered alone in an adjuvant capacity, may not mediate tumor regression as effectively as other cytokines. The reasons for a lack of optimal effectiveness of some cytokines are proffered herein: IL-4 may steer the T helper T cell population toward a Th2 phenotype responsible for the enhancement of humoral responses, but not of cell mediated immunity. IFN-γ, which upregulates the production of key molecules involved in antigen processing and presentation of intracellular antigens, may also have an anti-proliferative effect on T cells. For cytokines to serve as effective adjuvants resulting in active treatment to mediate tumor reduction in accordance with the present invention, they should function to cause the proliferation and activation of primed lymphocytes, particularly CTLs. However, increasing the concentrations of cytokines used, or employing combinations of different cytokines, including those mentioned above, may achieve an enhanced immune response against pathogenic diseases and cancer when used in conjuction with DNA-based immunization.

In a related aspect, DNA encoding one or more cytokines may be contained in the plasmid DNA comprising the immunizing DNA encoding the tumor or pathogenic disease-associated antigen, or immunodominant portion thereof, and under the control of the same or a different promoter and/or regulatory or control sequences. Further, DNA encoding one or more costimulatory agents may be contained in the plasmid DNA comprising the immunizing DNA encoding the tumor or pathogenic disease-associated antigen and under the control of the same or a different promoter or regulatory or control sequences. In this way, a mammal is immunized not only with DNA which

encodes proteins from the disease causing agent or tumor, but also with DNA which encodes molecules that stimulate the activation and proliferation of cytolytic T lymphocytes, which are needed to reduce or eradicate the disease or tumor. The effect of antigen-specific DNA immunization using cytokine DNA (and/or costimulatory molecule DNA) is similar to another of the invention's aspects which includes the administration of exogenous cytokines as adjuvants (and/or the ex-administration of exogenous costimulatory molecules) following (or at the time of) DNA immunization.

Cytokines as adjuvants may be administered by several modes and routes, such as parenterally, including intraperitoneal (i.p.), subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intrathecal, and intrasternal, for example. The cytokines are administered in a sterile solution or suspension, such as sterile physiological saline or other injectable aqueous liquids, or may be emulsified in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives and material for rendering the solution or suspension isotonic with body fluids (e.g., blood) of the recipient. Excipients suitable for use are water, phosphate buffered saline, pH 7.4, aqueous sodium chloride solution (0.15 M) dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. If oral administration is desired or necessary, the composition, including cytokine, may be presented as a draught in water or in a syrup, in capsules, cachets, boluses, or tablets, as will be known to those having skill in the art.

With regard to the safety of vaccination with nucleic acids, it has been shown to be difficult to induce antibodies against double-stranded DNA as immunogen (Van den Eynde, B., et al., 1991, The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. J. Exp. Med. 173:1373). In contrast, denatured single stranded DNA as an immunogen has the ability to induce an anti-DNA antibody response which potentially could result in an autoimmune disease such as systemic lupus erythematosus. The present invention, which preferably comprises double stranded DNA as immunogen, promises to be safe and is not expected to generate adverse

antibody responses, but rather to result in the generation of heightened antibody and cell-mediated responses that are specific for the disease causing antigens, as demonstrated by the experimental evidence herein.

EXAMPLES

The following examples are illustrative of the invention. They are presented to further facilitate an understanding of the inventive concepts and in no way are to be interpreted as limiting the scope of the present invention.

EXAMPLE 1

Example 1 describes the materials and methods employed in the further examples presented in Examples 2-6 hereinbelow.

Tumor Cell Lines and Animals

The murine colon adenocarcinoma, CT26.WT, is a clone of the N-nitroso-N-methylurethane induced BALB/c (H-2^d) undifferentiated colon carcinoma (Brattin, M. G., et al., 1980, Establishment of mouse colonic carcinoma cell lines with different metastatic properties. Cancer Res. 40:2142). Following transduction with a retrovirus encoding the Lac Z gene, CT26.WT was subcloned to generate the β-gal-expressing cell line CT26.CL25 (Wang, M., et al., 1995, Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J. Immunol. 154 (9):4685). All tumor cell lines were grown and maintained in complete medium (CM) containing RPMI 1640, 10% heat inactivated FCS (both from Biofluids, Rockville, MD), 0.03% fresh L-glutamine, and 100 μg/ml gentamicin sulfate. CT26.CL25 was grown in the presence of 400 μg/ml G418 (GIBCO, Grand Island, N.Y.). Female BALB/c mice, 6-10 weeks old, were obtained from the Animal Production Colonies, Frederick Cancer Research Facility, National Institutes of Health (Frederick, MD).

Plasmid Preparation

A plasmid vector, called WRG 3033, which encodes the E. coli Lac Z gene under the control of the human cytomegalovirus (CMV) intermediate-early promoter and designated pCMV/β-gal (Agracetus, Middleton, WI). It is to be understood that other similar and commercially-available plasmids containing the LacZ gene and suitable promoters, such as the CMV immediate-early promoter,

which is commercially available from Clontech, Palo Alto, CA) are appropriate for use in the DNA immunogens of the invention. Those having skill in the art are able to employ routine methods in the art to construct suitable plasmids for use in accordance with the invention. With particular regard to the construction of the above-mentioned pCMV/ß-gal vector, suitable for mammalian use, the CMV-ß-galactosidase portion of a CMV/ß-gal plasmid obtained from Clontech was produced by digestion of the CMV/ß-gal plasmid with *Eco*R1 and *Hind*III, thereby creating a fragment containing the CMV promoter and the LacZ coding region. This fragment was ligated into the PGEM 4Zf vector, commercially available from Promega Corp., Madison, WI, which had been restricted with *Eco*R1 and *Hind*III. Plasmids expressing either human growth hormone (pCMV/hGH) (provided by Agracetus, Middleton, WI or constructed employing commercially available or published nucleic acid sequences) or the nucleoprotein from influenza A (A/PR/8/34) (pCMV/NP) under the control of the CMV intermediate-early promoter were used as control vectors. As will be apparent to those skilled in the art, the control plasmid vectors are constructed using routine methods and reported nucleic acid sequence information for hGH and influenza A NP, for example. Constructs were transformed into E. coli DH5 alpha competent cells (GIBCO/BRL, Rockville, MD) and grown in L-broth supplemented to contain 100 μg/ml ampicillin as described (Sambrook, J., et al., 1989, Molecular Cloning: A Laboratory Manual, 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Closed circular plasmid DNA was isolated using Wizard Maxipreps DNA purification kits (Promega Corp., Madison, WI). The 260/280 ratios ranged from 1.8 to 2.0.

Peptides

The synthetic peptide β-gal$_{876-884}$, TPHPARIGL, (SEQ ID NO:1), representing the naturally processed H-2 L^d restricted epitope spanning amino acids 876-884 of β-gal (Gavin, M. A., et al., 1993, Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. J. Immunol. 151:3971) and the influenza $NP_{147-155}$ peptide, TYQRTRALV (SEQ ID NO:2), presented by H-$2K^d$ were synthesized by Peptide Technologies

(Washington, DC) to a purity of greater than 99% as assessed by HPLC and amino acid analysis.

Gene Gun Delivery of DNA

Plasmid DNA was affixed to gold particles by adding 10-50 mg of 0.95 μm gold powder (Agracetus, Middleton, WI) to 1.5 ml centrifuge tubes containing 100 μl of 0.1 M spermidine (Sigma Chemical Co., St. Louis, MO). Plasmid DNA and gold were coprecipitated by the addition of 200 μl of 2.5 M $CaCl_2$ during vortex mixing as previously described (Fuller, D.H., et al., 1995, A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune response in mice receiving a DNA-based glycoprotein 120 vaccine. AIDS Res. Hum. Retrovir. 10 (11):1433). After settling for ten minutes, the precipitate was washed with absolute ethanol to remove H_2O and resuspended at either 3.5 mg gold/ml or 7.0 mg/ml of ethanol that resulted in 0.25 mg or 0.5 mg of gold particles per shot, respectively. While the amount of gold per shot remained constant, the total amount of DNA per shot ranged from 0.001 - 1.0 μg/shot. Animals were anesthetized with 200 μl of a 9% solution of sodium pentobarbital while abdominal areas were shaved. DNA coated gold particles were delivered into abdominal epidermis using the hand-held, helium driven device Accell® gene delivery system (Agracetus, Middleton, WI, and commercially available from BioRad, Hercules, CA). Each animal received 2-10 non-overlapping deliveries per immunization (as designated below), at a pressure of 400 psi of helium.

Enzyme Linked Immunosorbant Assay (ELISA)

BALB/c mice were immunized two times at two week intervals with 0.001-1.0 μg of either pCMV/β-gal or pCMV/NP using the gene gun. Serum samples were collected two weeks following the second immunization and analyzed for the presence of anti-β-gal antibodies by ELISA. Specifically, microtiter plates were dried overnight at 37°C in a non-humidified incubator with 200 ng/well/50 μl of either purified β-gal or control antigen, ovalbumin (both obtained from Sigma Chemical Company, St. Louis, MO). One hundred μl of 5% bovine serum albumin (BSA) in PBS were incubated on each well for 1 hour to prevent

nonspecific Ab binding. This was followed by a second 1 hour incubation with 50 μl of five-fold dilutions (starting at 1:100) of test sera or control anti-β-gal murine mAb (starting at 100ng/50μl). After washing with 1% BSA in PBS, horseradish peroxidase conjugated sheep anti-mouse IgG F(ab)'$_2$ fragments (1:3000) (Amersham International, Amersham, UK) were added for 1 hour at 37°C to detect antibodies immobilized on the wells. The resulting complex was detected by the chromogen, o-phenylenediazamine (Sigma Chemical Company, St. Louis, MO). Absorbance was read on a Titertek Multiskan Plus reader (Flow Laboratories, McLean, VA) using a 490 nm filter. Concentrations of β-gal-specific antibody in serum samples were estimated from the mAb standard curve and expressed as μg/ml.

Effector Cells

Primary lymphocyte populations were generated by immunization with different amounts of purified pCMV/β-gal or control pCMV/NP. Secondary in vitro effector populations were generated by harvesting spleens of mice 14 days after immunization and culturing single cell suspensions of splenocytes in T-75 flasks (Nunc, Roskilde, Denmark) at a density of 3.0 x 10^6 cells/ml with 1 μg/ml antigenic peptide at a total volume of 30 ml of CM containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (both from Biofluids) and 5 x 10^{-5}M 2-mercaptoethanol (GIBCO BRL, Rockville, MD) in the absence of IL-2. Six days later, splenocytes were harvested and washed in CM before testing in a ^{51}Cr release assay or for transfer to tumor bearing animals.

^{51}Cr release assay

Six hour ^{51}Cr release assays were performed as previously described (Restifo, N.P., et al., 1993, Identification of human cancers deficient in antigen processing, J. Exp. Med. 177:265). Briefly, 2 x 10^6 target cells were incubated in 0.2 ml of CM labeled with 200 μCi of Na^{51}CrO$_4$ for 90 minutes. Peptide pulsed CT26.WT were incubated with 1 μg/ml (approximately 1 μM) of antigenic peptide during labeling as previously described (Restifo, N.P., et al., 1991, Defective presentation of endogenous antigens by a murine sarcoma: Implications for the failure of an anti-tumor immune response. J. Immunol. 147:1453). Target cells

were then mixed with effector cells for six hours at 37°C at the effector to target ratios indicated (Fig. 2A-2D). The amount of ^{51}Cr released was determined by γ (gamma) counting and the percentage of specific lysis was calculated as follows: % Specific lysis = [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] x 100.

<u>In vivo</u> **protection studies**

For <u>in vivo</u> prevention studies, mice were immunized with different amounts of pCMV/β-gal or with either control pCMV/hGH or pCMV/NP. Fourteen days later, mice were challenged i.v. with 2 x 10^5 tumor cells (Mulé, J.J., et al., 1987, Identification of cellular mechanisms operational <u>in vivo</u> during the regression of established pulmonary metastases by the systemic administration of high-dose recombinant interleukin-2. <u>J. Immunol.</u> 139:285). Mice were sacrificed on day 17 and randomized before counting lung metastases in a blinded fashion as previously described (<u>Ibid.</u>).

Adoptive Immunotherapy experiments

BALB/c mice were infected i.v. with 2 x 10^5 CT26.WT (β-gal -) or CT26.CL25 (β-gal +) cultured tumor cells in 0.5 ml of HBSS to induce pulmonary metastases. On day three, tumor bearing mice were treated with an i.v. injection of various effector cells at 5 x 10^6 cells/dose. Specifically, for the generation of effector cells, mice were immunized with 1 μg of pCMV/β-gal or pCMV/NP. Each mouse received two 0.25 mg shots of gold loaded with 0.5 μg DNA. Two weeks later, splenocytes were harvested and cultured for six days in CM plus 5 x 10^{-5} M 2-mercaptoethanol, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate with 1 μg/ml of either β-gal$_{876-884}$ (SEQ ID NO:1) or NP$_{147-155}$ (SEQ ID NO:2) synthetic peptide. On day 17 after tumor injection, mice were killed and pulmonary metastases were enumerated in a coded blinded fashion. When metastases exceeded 250, they were deemed too numerous to count.

<u>In vivo</u> **treatment studies**

BALB/c mice were challenged with 5 x 10^5 CT26.WT or CT26.CL25 tumor cells i.v. to establish pulmonary metastases. On day 2, mice were immunized with 10 μg of either pCMV/β-gal, pCMV/NP DNA or no DNA.

Each mouse received ten non-overlapping shots of gold (0.5 mg each) delivering 1 μg of DNA. On day 12, the mice were killed and metastatic lung modules were enumerated in a randomized, blinded manner. As a positive control, a group of mice was included that received a recombinant vaccinia virus encoding β-gal (VJS6) plus the exogenous administration of the cytokine IL-2 (15,000U, BID, for 5 days) as previously reported (Bronte, V., et al., 1995, IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases. J. Immunol. 154 (10):5282).

Intraperitoneal treatments of various cytokines began 18-24 hours following DNA administration and continued daily for 3-7 days depending on the cytokine. Specifically, one group of mice received 15,000 cetus units of recombinant human IL-2 (rhIL-2) twice daily (BID) for five days (Chiron Corp., Emeryville, CA) (Ibid.). A second group of mice received treatments of 0.5 μg of recombinant mouse IL-6, BID, for three days (Peprotech, Inc., Rocky Hill, NJ). A third group received 5 μg of rhIL-7 for seven days (Peprotech, Inc., Rocky Hill, NJ). A fourth group received 1.0 μg of rmIL-12 once daily (QD) for five days (Genetics Institute, Boston, MA). In a screening experiment of many cytokines, rmIL-4 (5μg, BID, 7 days), rmIL-10 (1.0 μg, QD, 7 days), and GM-CSF (1.0 μg, QD, 5 days) were also assayed for adjuvant activity (Peprotech, Rocky Hill, NJ). These parameters were determined from previous reports demonstrating anti-tumor immune responses from their exogenous administration (Jicha, D. L., et al., 1991, Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. J. Exp. Med. 174:1511; Komschlies, K. L., et al., 1994, Administration of recombinant human IL-7 to mice alters the composition of B-lineage cells and T cell subsets, enhances T cell function, and induces regress of established metastases. J. Immunol. 153:5776; Mulé, J.J., et al., 1992, Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol. 148:2622; Brunda, M.J., et al., 1993, Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 178:1223; Hill, A.D., et al., 1993, Granulocyte-macrophage colony-stimulating factor inhibits tumor growth. *Br. J. Surg. 80 (12)*:1543; and Topp,

M.S., et al. 1993. Recombinant human interleukin-4 inhibits growth of some human lung tumor cell lines in vitro and in vivo. Blood 82:2837). These doses of cytokine alone were shown to have little or no effect on the growth of CT26.WT or of CT26.CL25.

Statistical analysis

Because lungs that contained >250 pulmonary metastases were deemed too numerous to count, the data do not follow a normal distribution. Thus, statistical evaluation of the data was performed using the non-parametric two tailed Kruskal-Wallis test.

EXAMPLE 2

DNA-based vaccines elicit antigen specific humoral and cellular immunity

To demonstrate the induction of antigen-specific humoral immunity using a DNA-based vaccine, mice were immunized with a plasmid cDNA encoding the model TAA β-gal (pCMV/β-gal). Mice immunized and boosted with gold particles coated with as little as 0.01 μg of pCMV/β-gal developed β-gal specific antibody responses (Fig. 1). In contrast, gold particles coated with 1.0 μg of the control plasmid pCMV/NP failed to elicit a β-gal specific antibody response. In addition, no reactivity was observed against a control antigen, ovalbumin, confirming the specificity of the humoral immune response.

To assess whether a CTL response was elicited against β-gal expressed by DNA immunization, BALB/c mice were inoculated one time with varying quantities of pCMV/β-gal or control vector, pCMV/NP. Splenocytes from mice that were injected with as little as 0.1 μg of pCMV/β-gal and then restimulated in vitro with the β-gal$_{876-884}$ (SEQ ID NO:1) peptide lysed the β-gal transfected tumor cells, CT26.CL25 (Fig. 2A-2D). CT26.WT cells pulsed with the immunodominant peptide β-gal$_{876-884}$ (SEQ ID NO:1) was also recognized by the pCMV/β-gal immune splenocytes generated with at little as 0.01 μg of DNA. Pulsed cells in this case appeared to be more sensitive to lytic cells than the transfected cells, CT26.CL25. Unpulsed CT26.WT cells were not significantly lysed, thus demonstrating the specificity of the lytic response. Splenocytes derived from mice immunized with pCMV/NP and cultured in vitro in a manner identical

to that described above failed to lyse CT26.WT, CT26.WT pulsed with the β-$gal_{876-884}$ peptide (SEQ ID NO:1), or CT26.CL25. Together, these data indicated that DNA administered with the gene gun was able to elicit specific humoral and T_{CD8+} cellular immunity against the model tumor antigen, β-gal.

EXAMPLE 3

Prophylactic DNA vaccine protects mice from intravenous tumor challenge

To determine whether humoral and cellular responses observed in vitro correlated with in vivo antitumor activity, mice were immunized with the DNA vaccines of the invention and assayed for the growth of a subsequent intravenous tumor challenge. Only the mice that had received pCMV/β-gal as immunogen showed significant responses when compared with the mice that were inoculated with control DNA (Fig. 3). In pooled results from three experiments, virtually complete protection was observed with 1.0 μg of pCMV/β-gal immunogen; 19 out of 20 pairs of lungs from immunized mice were devoid of any detectable tumor. At a 10-fold lower dose, 16 out of 20 pairs of lungs were completely free of disease. With a dose of 0.01 μg of DNA immunized, the protective effects began to wane, with only 6 out of 20 mice remaining disease free. These results correlated with decreasing amounts of antibody and CTL activity observed in mice that received 0.01 μg of DNA (Figs. 1 and 2A-2D). In addition, mice immunized with pCMV/β-gal DNA immunogen and then challenged with β-gal negative-CT26.WT were not protected from tumor growth (Fig. 3). Therefore, these results indicate that the protection from tumor following gene gun inoculation of DNA encoding a TAA is antigen specific, both at the level of the immunizing plasmid DNA and at the level of antigen expression by the tumor.

EXAMPLE 4

Adoptive transfer of splenocytes generated using a DNA vaccine mediated the reduction of established pulmonary metastases

To ascertain whether the effector cells elicited by gene gun immunization were active in in vivo adoptive immunotherapy, a three-day lung metastasis model was used (Mulé, J.J., et al., 1987, Identification of cellular mechanisms operational in vivo during the regression of established pulmonary

metastases by the systemic administration of high-dose recombinant interleukin-2. J. Immunol. 139:285). In these experiments, BALB/c mice were given i.v. injections with either CT26.CL25 (β-gal +) or CT26.WT (β-gal -) tumor cells. On day three, tumor bearing mice were treated with splenocytes from donor mice that had received gene gun immunization with either pCMV/β-gal DNA as immunogen or pCMV/NP DNA as immunogen, and then subsequent ex vivo incubation of the cells with either β-gal$_{876-884}$ (SEQ ID NO:1) or control NP$_{147-155}$ (SEQ ID NO:2) peptide.

Effector cells generated from mice immunized with pCMV/β-gal and cultured with β-gal$_{876-884}$ (SEQ ID NO:1) peptide completely cleared the lungs of mice bearing 3 day old pulmonary metastases (Fig. 4). By contrast, lungs from CT26.CL25 bearing mice that received either no splenocytes, splenocytes from pCMV/β-gal immunized mice stimulated with the irrelevant synthetic peptide, NP$_{147-155}$ (SEQ ID NO:2), or splenocytes from pCMV/NP immunized mice stimulated with the β-gal$_{876-884}$ (SEQ ID NO:1) peptide contained >250 pulmonary metastases. In addition, the splenocytes induced by pCMV/β-gal DNA and stimulated with β-gal$_{876-884}$ (SEQ ID NO:1) peptide were not effective at eliminating metastases in mice bearing the antigen-negative tumor, CT26.WT (Fig. 4), thus demonstrating the in vivo specificity for the β-gal antigen. Similar results were observed in a repeat experiment. Thus, the induction of immune splenocytes for adoptive transfer was antigen specific, and the therapeutic activity of these cells was specific for antigen expression by the tumor. The preventive and therapeutic implications of this approach are clear, since this strategy involved not only the prevention of disease, but also the treatment of established metastases.

EXAMPLE 5

Gene gun immunization of DNA alone assessed for specific immunotherapy of established pulmonary metastases

Mice bearing two day established pulmonary metastases were immunized with pCMV/β-gal to evaluate the ability of gene gun immunization of DNA to generate active specific therapeutic responses. When tumor bearing mice were given up to 10μg of DNA utilizing the gene gun, no impact on tumor burden

was observed. Tumor bearing mice also were not treated when 100μg of DNA was administered intramuscularly. A control recombinant vaccinia virus, VJS6, followed by rhIL-2 administration did diminish the number of nodules to an average of 83 metastases per lung, as previously observed (Bronte, V., et al., 1995, IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases. J. Immunol. 154 (10):5282). One out of seven independent experiments using gene gun immunization alone in the absence of cytokine administration to treat established pulmonary metastases yielded a reduction in tumor growth (Fig. 6). It is possible that higher amounts of DNA immunogen (i.e., >100μg) reduce tumor burden, in the absence of cytokine co-administration following inoculation with immunogen.

EXAMPLE 6

Cytokine administration following DNA vaccine leads to treatment of established pulmonary metastases

Several cytokines, known to have different immune regulating effects, have been reported to have antitumor activity as a single agent therapy (Komschlies, K.L., et al., 1994, Administration of recombinant human IL-7 to mice alters the composition of B-lineage cells and T cell subsets, enhances T cell function, and induces regress of established metastases. J. Immunol. 153:5776; Mulé, J.J., et al., 1992, Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol. 148:2622; Brunda, M.J., et al., 1993, Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 178:1223; and Tahara, H., et al., 1995, Antitumor effects of interleukin-12 (IL-12): applications for the immunotherapy and gene therapy of cancer. Gene Ther. 2(2):96), as well as serving as adjuvant to standard therapy (Komschlies, K.L., et al., 1994, Administration of recombinant human IL-7 to mice alters the composition of B-lineage cells and T cell subsets, enhances T cell function, and induces regress of established metastases. J. Immunol. 153:5776; Takatsuki, F., et al., 1988, Human IL-6/B cell stimulatory factor-2 augments murine antigen-specific antibody responses in vitro and in vivo. J. Immunol. 141:3072; Afonso, L.C., et al., 1994, The adjuvant effect of interleukin-12 in a

vaccine against Leishmania major. Science 263(5144):235; and Noguchi, Y., et al., 1995, Influence of interleukin 12 on p53 peptide vaccination against established Meth A sarcoma. Proc. Natl. Acad. Sci. USA 92:2219). For example, rhIL-2, rmIL-6 and rhIL-7 have been reported to activate or proliferate antigen-specific CTL populations (Mulé, J.J., et al., 1992, Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol. 148:2622; Jicha, D.L., et al., 1991, Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. J. Exp. Med. 174:1511). GM-CSF promotes the differentiation of hematopoietic precursors to dendritic cells that function to present antigen to prime naive lymphocytes (Inaba, K., et al., 1992, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176:1693). rmIL-12, rmIL-4 and rmIL-10 have been shown to direct T helper populations to different Th1 or Th2 phenotypes resulting in shifts to either humoral or cell mediated responses, respectively (Mehrona, P.T., et al., 1993, Effects of IL-12 on the generation of cytotoxic activity on human CD8+ T lymphocytes. J. Immunol. 151 (5):2444; Hsieh, C.S., et al., 1993, Development of Th1 CD4+ T cells through IL-12 produced Listeria-induced macrophages. Science 260:547; Bertagnolli, M.M., et al., 1992, IL-12 augments antigen-dependent proliferation of activated T lymphocytes. J. Immunol. 149:3778; Sypek, J.P., et al., 1993, Resolution of cutaneous Leishmaniasis: Interleukin 12 initiates a protective T helper Type 1 immune response. J. Exp. Med. 177:1797). A screening assay was performed to determine whether various cytokines acted as adjuvants to DNA vaccination to induce active specific immunotherapy. In these experiments, tumor bearing mice immunized on day 2 with pCMV/β-gal or unimmunized were treated 18-24 hours later with either rhIL-2, rmIL-4, rmIL-6, rhIL-7, rmIL-10, rmIL-12 or rmGM-CSF administered exogenously. From this screening assay, rhIL-2, rmIL-6, rhIL-7 and rmIL-12 were found to specifically and optimally induce antigen-specific active immunotherapy.

Fig. 5 illustrates the average number of pulmonary metastases on day 12 following tumor challenge for those mice that received the DNA vaccine

followed by either rhIL-2, rmIL-6 or rhIL-7 administration. Fig. 5 represents the pooled averages of two separate experiments performed using the same protocol. Mice that were injected with pCMV/β-gal DNA immunogen alone demonstrated no significant reduction in the number of metastases (215), compared with control non-immunized mice (>250). The groups of mice that received pCMV/β-gal administration plus the individual cytokines rhIL-2, rmIL-6 or rhIL-7 all demonstrated a significant reduction in the number of metastases compared with either cytokine alone (all >250, p_2=0.001, p_2=0.003 and p_2=0.0002, respectively), β-gal alone (>250, p_2=0.003, p_2=0.0003 and p_2=0.001, respectively.

In an extensive experiment particularly evaluating the effects of IL-12 as adjuvant, mice treated (immunized) with pCMV/β-gal DNA alone showed a slight but significant reduction in the number of pulmonary metastases compared with the group that received pCMV/NP (Fig. 6; i.e., 151 compared with 239, p_2=0.016). This result was observed in one experiment out of seven experiments performed. rmIL-12 used alone demonstrated only a modest reduction in the tumor burden with an average of 177±45 metastatic nodules (mets) compared with >250 in the untreated group. However, a dramatic reduction was observed using the combination of pCMV/β-gal DNA and rmIL-12 (an average of 8±2.5 mets) (Fig. 6). This was significant compared with groups that received pCMV/β-gal DNA alone (p_2=0.012), rmIL-12 alone (p_2=0.002), and pCMV/NP plus rmIL-12 (p_2=0.008). pCMV/β-gal resulted in a significant reduction in tumor burden even with lower doses of rmIL-12. Doses of 0.3 and 0.1 μg of rmIL-12 were also able to diminish the tumor burden to 17±3.8 and 32±9.2 metastases, respectively. These results were significant compared with controls, i.e., pCMV/β-gal alone (p_2=0.036 and p_2=0.036, respectively) and rmIL-12 alone (p_2=0.002 and p_2=0.004, respectively). Therefore, when rmIL-12 is administered as an adjuvant with pCMV/β-gal DNA, lower doses of rmIL-12 can be given and therapeutic effects are still observed. This kind of active treatment experiment using IL-12 as an adjuvant to DNA immunization has been repeated three times with similar findings. Together, these studies show that cytokines IL-2, IL-6, IL-7 and IL-2,

given individually and in combination with pCMV/β-gal, provide antigen specific reduction in tumor burden. It is also envisioned that combinations of the different cytokines (or one or more) will be useful in reducing disease and tumors in an antigen-specific manner.

EXAMPLE 7

Clinical protocol for Phase I/II trials in patients with metastatic melanoma for nucleic acid-based immunization with DNA encoding MART-1 and cytokine administered as adjuvant

In this protocol, patients with advanced melanoma are immunized against MART-1, an immunodominant protein from a cancer antigen, in conjunction with cytokine as adjuvant as described herein. In addition, costimulatory/accessory molecules, such as B7-1, also as described herein, may be used in combination with DNA immunization and cytokine administration.

Patient eligibility Among other criteria, patients must have evidence of measurable or evaluable metastatic melanoma that has failed standard effective therapy. Patients must have tumors that express the MART-1 antigen as evidenced by PCR or Northern Blot analysis of tumor cell RNA.

Vaccine/Immunogen preparation The DNA-based vaccine/immunogen used in this protocol is a PUC-1-based plasmid containing the gene coding for the MART-1 protein under the control of the CMV promoter, and also having the CMV intron A enhancer region and splice donor/acceptor sites. The DNA-based immunogen is produced in a Food and Drug Administration (FDA) approved facility for the manufacture of GMP grade clinical material.

Vaccination procedure The DNA for vaccination will be precipitated onto gold pellets which are coated and dried onto the interior of Teflon tubing (e.g., Teszel Tubing, 1/8 inches OD; 3/32 inches ID; McMasters Carr). Approximately half-inch pieces of the gold-coated tubing are used as "bullets". Within each bullet is approximately 0.25 to 0.5 mg of gold which carries from 0.001 μg to as much as 1 μg of DNA. The size of the gold pellets are determined according to need and use and can range from about 0.95 microns to about 1 to 3 microns.

Method of vaccination and treatment plan The method of vaccination and

treatment plan are summarized as follows:

1. Melanoma patients receive epidermal immunization with plasmid cDNA encoding MART-1 driven by the CMV promoter as described above and approved by the FDA. Patients receive three successive immunizations, each separated by three to four weeks with the same dose of DNA. Cohorts of patents are treated at each dose. Patients will be observed for 24 hours after immunization. Body temperature will be measured at 12 hours and at 24 hours and any adverse reactions noted.

2. The dose escalation schedule for the MART-1 DNA immunization (μg DNA) is approximately as follows:

1. 0.4 μg
2. 2.0 μg
3. 10 μg
4. 20 μg

3. The adjuvant cytokine is administered 18 to 24 hours following DNA vaccination.

4. Doses of 0.001-10 μg are well tolerated by a 20 gram mouse and are thus 1/30000 of the dose by weight tolerated by mice. Patients are carefully evaluated for toxicity. When three patients at each dose level have been followed for at least two weeks after the first immunization without achieving grade III or IV toxicity, not easily reversible by standard measures, then the dose in that patient category will be escalated to the next dose level. Patients are also monitored for anti-DNA antibodies. In addition, cells at the site of injection are assayed for potential DNA integration, a potential problem, but one that has not previously been observed in the mouse model system when DNA was directly injected into muscle. Expected accruel is 15-20 patients a year.

5. At the MTD of DNA vaccination, three patients are vaccinated with DNA immunogen, followed by IL-2 or IL-12 administration, in each of the following groups:

a) IL-2, subcutaneous injection of 250, IU/kg daily for 5 days and repeated once after a 2 day rest.

b) IL-2, intravenous infusion of 720,000 IU/kg every 8 hours

for 7 days, not to exceed 21 doses.

c) IL-2, intravenous doses of 720,000 IU/kg every 8 hours for 5 days not to exceed 15 doses.

d) IL-12, intravenous doses in amounts determined in consideration of murine studies in which mice were given 0.5 μg/mouse once a day for 5 days.

6. Based upon the findings of initial immunologic studies, up to 15 patients will be treated at a single dose level that results in evidence of cell mediated immune effects to confirm the immunologic findings.

The accumulation of biologic data concerning the development of cellular immune responses to the MART-1 antigen is the primary goal of treating these 15 patients. If any patient at any dose level achieves grade III or grade IV toxicity, then an additional three patients will be treated at that dose. If a second patient develops grade III or grade IV toxicity not easily managed by standard procedures, then the dose will not be escalated.

Interleukin-2 (IL-2) administration

IL-2 is administered at a dose of 720,000 IU/kg as an intravenous bolus over a 15 minute period every eight hours beginning on the day after DNA immunization and continuing for a total of four days. Doses may be skipped depending on patient tolerance. For example, doses are skipped if patients reach grade III or IV toxicity. If this toxicity is easily reversed by supportive measures, then additional doses may be given. No more than 12 doses of IL-2 are administered after each immunization. IL-2 is administered as an inpatient.

IL-2 Formulation/Reconstitution: IL-2 (Chiron), NSC #373364, is provided as a lyophilized powder. Each 5 cc vial has a labeled strength of 1.3 mg (22 Million IU). The vial is reconstituted with 1.2 ml of sterile water for injection, USP, and the resultant concentration is 18 million IU/ml. Diluent should be directed against the side of the vial to avoid excess foaming. Vial contents are swirled, not shaken, gently until completely dissolved. Since vials contain no preservative, the reconstituted solution should be used within 8 hours.

Storage: Intact vials are stored in the refrigerator (i.e., 2°C-8°C)

with protection from light. Each vial bears an expiration date.

Dilution/Stability: Reconstituted IL-2 is further diluted with 5% Dextrose, USP and is not mixed with saline-containing solutions. Reconstituted IL-2 may be diluted as necessary in volumes of 50 ml to 500 ml with 5% Dextrose, USP plus 0.1% Albumin Human, USP. When diluting, the Albumin Human, USP should be added to the 5% Dextrose Injection, USP prior to the addition of the IL-2. When diluted for IV administration in 5% Dextrose Injection, USP, in a plastic bag (e.g. Viaflex, manufactured by Travenol Laboratories, Inc.,) containing 0.1% Albumin Human, IL-2 is chemically stable for 48 hours at both refrigerated and room temperatures, (i.e., 2°C-30°C).

Subcutaneous administration of IL-2

Patients begin receiving IL-2 by daily subcutaneous injection at a dose of 250,000 IU/kg on days 1-5 of therapy. After resting on days 6 and 7, patients then receive 5 daily injections as a second cycle of 250,000 IU/kg per dose. Therapy begins as an inpatient with teaching of patients to perform self-administered subcutaneous injections. Once a patient is judged competent to reconstitute and self-inject (or a family member is considered competent), the patient is discharged to home to continue therapy. IL-2 is provided as lyophilized powder to be reconstituted by the patient with 1.2 cc of sterile water (U.S.P.). All reconstituted vials must be kept refrigerated (5°C) and must be discarded after 24 hours. All patients receive tylenol 650 mg p.o., every 4-6 hours while on therapy and indocin 50 mg p.o. every 6 hours is used on a PRN basis to treat constitutional symptoms.

Because confusion is a possible side effect of IL-2 administration, a Durable Power of Attorney is signed by patients receiving IL-2 to identify a surrogate to make decisions, if a patient becomes unable to make decisions. As necessary or required during the period of IL-2 administration, patients are treated as in-patients and have vital signs monitored every 4 hours and are seen by physicians at least twice per day to monitor for confusion.

Concomitant therapy All patients receiving IL-2 receive concomitant medications to relieve side effects, as is routine in high-dose IL-2 protocols. The following

concomitant medication should begin the evening before the first dose of IL-2 and continue throughout the entire course of treatment: acetaminophen (650 mg every 4 hours), indomethacin (50-75 mg every 6 hours), and ranitidine (150 mg every 12 hours). Patients may receive intravenous meperidine (25 to 50 mg) to control chills when they occur, although chills are unusual after the first one to two doses of IL-2. Steroids are not used in these patients and if steroids are required, then the patient is removed from the protocol.

Patient evaluation

Pretreatment Prior to the DNA vaccination and treatment protocol, patients will be evaluated and the following tests performed:

a) Complete history and physical examination, including exact size and location of any measurable neoplastic disease.

b) Acute care, hepatic, mineral and thyroid blood chemistry panel.

c) Complete blood count with differential.

d) Prothrombin time and partial thromboplastin time.

e) Platelet count.

f) Urine analysis.

g) HIV antibody titer and Hepatitis B antigen determination.

h) Chest x-ray and electrocardiogram

i) All females of reproductive potential must have a negative pregnancy test.

j) Baseline x-rays and nuclear medicine scans to evaluate the status of disease.

k) HLA typing by the NIH Clinical Center, HLA testing laboratory.

l) Histologic confirmation of the diagnosis of melanoma by pathologists at the NIH.

m) 10 ml of serum are stored at -20°C for subsequent testing.

n) Lymphocytes are separated by Ficoll gradients from 30 ml of whole blood and cryopreserved for subsequent immunologic testing. (Some patients may undergo a limited 2 to 3 hour leukapheresis to obtain additional lymphocytes at pretreatment and at one additional time after vaccination).

Day of immunization

Patients are observed for 24 hours after DNA immunization. Body temperature is measured at 6 to 12 hours, and again at 24 hours, and any adverse reactions are noted.

Post immunization evaluation

On day 14 after immunization the following patient information and tests will be obtained:

a) History, including any symptoms resulting from the immunization.

b) Examination of the immunization site if abnormal.

c) Complete blood count with differential count.

d) Platelet counts.

e) Acute care, peptic, mineral and thyroid blood chemistry panel.

f) Serum and lymphocytes cryopreserved as performed at pretreatment.

Patients are seen again on day 28 and the above tests are performed immediately prior to the second immunization. Immunization is repeated only if all toxicity has resolved to grade I or less. Patients are evaluated at 14 days following the second immunization, and similar procedures are performed prior to and following the third and final immunization.

Prior to each immunization, patients undergo an ophthalmologic examination to assess possible autoimmune ophthalmologic toxicity which, if present, is considered to be a dose-limiting toxicity. Patients have a complete restaging of all sites of disease with appropriate physical examination and X-rays and nuclear medicine studies at the time of two months following the final vaccination.

Immunologic studies

Immunologic assessment will be made of the patient's response to the MART-1 antigen, as follows:

a) Serum samples will be tested for anti-MART-1 antibody, e.g., by ELISA.

b) Cryopreserved lymphocytes will be tested for response to the MART-1 associated antigen (or GP-100 associated antigen, if applicable; see below) using limiting dilution analysis of precursor CTL frequency using the method of Coulie, P. et al., 1992, International Journal of Cancer 50:289-297.

c) Patients will easily accessible disease may have biopsy under local anesthesia of accessible tumor to study the histopathologic nature of the tumor, as well as the isolation of tumor infiltrating lymphocytes (TIL) for in vitro growth. TIL will be tested for specific reactivity and specific cytokine release against the MART-1 associated antigen.

Assessment of response

A complete response is defined as the disappearance of all clinical evidence of disease that lasts at least four weeks. A partial response is a 50% or greater decrease in the sum of the products of the perpendicular diameter of all measurable lesions for at least four weeks, with no appearance of new lesions or increase in any lesions. Minor responses are defined as about a 25% to 49% decrease in the sum of the products of the perpendicular diameters of all measurable lesions with no appearance of new lesions and no increase in any lesions. Any patient with less than a partial response is considered a non-responder. The appearance of new lesions, or greater than 25% increase in the product of perpendicular diameters of prior lesions following a partial or complete response, will be considered as a relapse.

Similar protocols will be followed for the evaluation of other DNA-based vaccines against other human cancers and tumors, including genetically predisposed or inherited cancers and tumors. Vaccines to be tested include, but are not limited to, immunogens comprising DNA encoding MART-1, followed by the systemic administration of one or more cytokines, preferably IL-6, IL-7, or IL-12, or combinations thereof; immunogens comprising DNA encoding MART-1 and DNA encoding one or more of the above-listed cytokines, with IL-12 preferred; immunogens comprising DNA encoding MART-1 and DNA encoding B7.1, followed by the administration of one or more cytokines, preferably IL-2, IL-6, IL-7, or IL-12, or combinations thereof, with IL-12 preferred; plasmid DNA

comprising DNA encoding MART-1, DNA encoding one or more cytokines with DNA encoding IL-2, IL-6, IL-7, or IL-12, or combinations thereof, preferred, in combinaion with DNA encoding B7.1; immunogen comprising DNA encoding GP100, followed by the administration of one or more cytokines, preferably IL-2, IL-6, IL-7, or IL-12, or combinations thereof, immunogens comprising plasmid DNA comprising DNA encoding GP100 and DNA encoding one or more of the above-listed cytokines, with IL-12 preferred; immunogens comprising DNA encoding GP100 and DNA encoding B7.1, followed by the administration of one or more cytokines, preferably IL-2, IL-6, IL-7, or IL-12, or combinations thereof, with IL-12 preferred; plasmid DNA comprising DNA encoding GP100, DNA encoding one or more cytokines, preferably, IL-2, IL-6, IL-7, or IL-12, or combinations thereof, and DNA encoding B7.1; and the like, immunogens comprising DNA encoding MAGE-1, followed by the systemic administration of one or more cytokines, preferably, IL-2, IL-6, IL-7, or IL-12, or combinations thereof; immunogens comprising DNA encoding MAGE-1 (for patients afflicted with melanoma and/or breast cancer) and DNA encoding one or more of the above-listed cytokines, with IL-12 preferred; immunogens comprising DNA encoding MAGE-1 and DNA encoding B7.1, followed by the administration of one or more cytokines, with IL-2, IL-6, IL-7, or IL-12, or combinations thereof preferred, and with IL-12 most preferred; plasmid DNA comprising DNA encoding MAGE-1, DNA encoding one or more cytokines, preferably IL-2, IL-6, IL-7, or IL-12, or combinations thereof, and DNA encoding B7.1, with the appropriate modifications depending on the DNA immunogen, the antigen, and the cytokine and/or costimulatory molecule used.

The contents of all patent applications, issued patents, published articles and references, and textbooks as cited herein are hereby incorporated by reference in their entirety.

As various changes can be made in the above compositions and methods without departing from the scope and spirit of the invention, it is intended that all subject matter contained in the above description, shown in the accompanying drawings, or defined in the appended claims will be interpreted as illustrative, and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE GOVERNMENT OF THE UNITED STATES AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES

(ii) TITLE OF INVENTION: CYTOKINE ENHANCEMENT OF DNA IMMUNIZATION RESULTS IN INCREASED SPECIFIC CELLULAR AND HUMORAL IMMUNE RESPONSES AGAINST PATHOGENIC DISEASES AND CANCER

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MORGAN AND FINNEGAN, L.L.P.
(B) STREET: 345 PARK AVENUE
(C) CITY: NEW YORK
(D) STATE: NEW YORK
(E) COUNTRY: USA
(F) ZIP: 10154-0053

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: FLOPPY DISK, 3.5"
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: TO BE ASSIGNED
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: LESLIE A. SERUNIAN
(B) REGISTRATION NUMBER: 35,353
(C) REFERENCE/DOCKET NUMBER: 2026-4213PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 758-4800
(B) TELEFAX: (212) 751-6849

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 AMINO ACIDS
(B) TYPE: AMINO ACID
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ESCHERICHIA COLI

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Pro His Pro Ala Arg Ile Gly Leu
1 5

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 AMINO ACIDS
(B) TYPE: AMINO ACID
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(vi) ORIGINAL SOURCE:
(A) ORGANISM: INFLUENZA VIRUS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Tyr Gln Arg Thr Arg Ala Leu Val
1 5

WHAT IS CLAIMED IS:

1. A nucleic acid immunogen comprising one or more isolated nucleic acid molecules encoding an antigen, or an immunogenic portion thereof, of a disease-causing agent, and one or more isolated nucleic acid molecules encoding a cytokine, said immunogen delivered to cells using direct gene delivery.

2. The immunogen according to claim 1, further comprising one or more isolated nucleic acid molecules encoding an immunostimulatory molecule.

3. The immunogen according to claim 1, wherein the disease-causing agent is a cancer or a pathogenic microorganism.

4. The immunogen according to claim 3, wherein the cancer is selected from the group consisting of non-Hodgkins lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, carcinoma, neuroblastoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

5. The immunogen according to claim 4, wherein the antigen is a tumor associated antigen.

6. The immunogen according to claim 5, wherein the tumor associated antigen is selected from the group consisting of oncofetal antigens, MART-1, MAGE-1, MAGE-3, GP-100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15 and β-galactosidase.

7. The immunogen according to claim 3, wherein the pathogenic microorganism is a virus, bacterium, protozoan, or yeast.

8. The immunogen according to claim 7, wherein the pathogenic

virus is HIV, hepatitis virus, human papillomavirus, equine encephalitis virus, herpes simplex virus or influenza virus.

9. The immunogen according to claim 1 or claim 2, wherein the cytokine or immunostimulatory molecule is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, B7.1, B7.2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, RANTES, and combinations thereof.

10. The immunogen according to claim 9, wherein the cytokine is selected from the group consisting of IL-2, IL-6, IL-7, and IL-12.

11. The immunogen according to claim 1, wherein the nucleic acid immunogen is delivered by means of particle bombardment or a gene gun device.

12. The immunogen according to claim 1, wherein the nucleic acid is affixed to gold particles.

13. The immunogen according to claim 12, wherein the gold particles are delivered by means of particle bombardment or a gene gun device.

14. The immunogen according to claim 11, 12, or 13, wherein the immunogen is delivered into the adominal epidermis.

15. A nucleic acid immunogen comprising one or more isolated nucleic acid molecules encoding a tumor associated antigen and one or more isolated nucleic acid molecules encoding a cytokine, said immunogen delivered to cells using direct gene delivery.

16. The immunogen according to claim 15, further comprising one or more isolated nucleic acid molecules encoding an immunostimulatory molecule.

17. The immunogen according to claim 15, wherein the tumor associated antigen is selected from the group consisting of oncofetal antigen, MART-1, MAGE-1, MAGE-3, GP-100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15, and β-galactosidase.

18. The immunogen according to claim 15 or claim 16, wherein the cytokine or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

19. The immunogen according to claim 18, wherein the cytokine is selected from the group consisting of IL-2, IL-6, IL-7, and IL-12.

20. The immunogen according to claim 15, wherein the nucleic acid immunogen is delivered by means of particle bombardment or a gene gun device.

21. The immunogen according to claim 15, wherein the nucleic acid is affixed to gold particles.

22. The immunogen according to claim 21, wherein the gold particles are delivered by means of particle bombardment or a gene gun device.

23. The immunogen according to claim 20, 21, or 22, wherein the immunogen is delivered into the adominal epidermis.

24. A method of preventing or treating a disease or cancer in a patient, comprising:

(a) introducing into the patient by direct gene delivery an effective amount of a nucleic acid immunogen comprising one or more isolated nucleic acid molecules encoding an antigen or an immunodominant epitope of a disease-causing agent or cancer; and

(b) administering to the patient a cytokine in an amount effective for enhancing the immune response of the patient and ameliorating or reducing the effects of the disease or cancer.

25. The method according to claim 24, further comprising the step of:

(c) administering to the patient an immunostimulatory molecule in an amount effective for enhancing or modulating the immune response of the patient.

26. The method according to claim 24, wherein the cytokine is encoded by cytokine-specific DNA and administered by means of direct gene delivery or the cytokine is exogenously administered to the patient.

27. The method according to claim 25, wherein the cancer is selected from the group consisting of non-Hodgkins lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, neuroblastoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

28. The method according to claim 25, wherein the disease is caused by a pathogenic microorganism selected from the group consisting of viruses, bacteria, protozoans, parasites, and yeast.

29. The method according to claim 25, wherein, in the introducing step (a), the nucleic acid immunogen is affixed to gold particles.

30. The method according to claim 24 or claim 29, wherein the nucleic acid is delivered by means of a gene gun device.

31. The method according to claim 30, wherein the nucleic acid is delivered into the abdominal epidermis.

32. The method according to claim 24 or claim 25, wherein the cytokine or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

33. The method according to claim 32, wherein the cytokine is selected from the group consisting of IL-2, IL-6, IL-7, and IL-12.

34. A method of preventing or treating a disease or cancer in a mammal comprising introducing into the mammal an effective amount of the immunogen according to claim 1, 2, 15, or 16, the amount effective in preventing, reducing or ameliorating the disease or cancer.

35. The method according to claim 34, wherein the cancer is selected from the group consisting of non-Hodgkins lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, neuroblastoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

36. The method according to claim 34, wherein the disease is caused by a pathogenic microorganism selected from the group consisting of viruses, bacteria, protozoans, parasites, and yeast.

37. The method according to claim 34, wherein the introduced nucleic acid immunogen is affixed to gold particles.

38. The method according to claim 34, wherein the nucleic acid immunogen is delivered by means of a gene gun device.

39. The method according to claim 38, wherein the nucleic acid immunogen is delivered into the abdominal epidermis.

40. The method according to claim 34, wherein the cytokine or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

41. The method according to claim 40, wherein the cytokine is selected from the group consisting of IL-2, IL-6, IL-7, and IL-12.

42. A pharmaceutical composition comprising the nucleic acid immunogen according to claim 1, alone or in combination with an exogenous immunostimulatory molecule, chemotherapy drug, antibiotic, antifungal drug, antiviral drug or combination thereof and a pharmaceutically acceptable carrier or excipient.

43. The pharmaceutical composition according to claim 42, wherein the cytokine or immunostimulatory molecule is selected from the group

consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

44. A pharmaceutical composition comprising the nucleic acid immunogen according to claim 15, alone or in combination with an exogenous immunostimulatory molecule, chemotherapy drug, antibiotic, antifungal drug, antiviral drug or combination thereof and a pharmaceutically acceptable carrier or excipient.

45. The pharmaceutical composition according to claim 44, wherein the cytokine or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

46. A method of preventing or treating a cancer or disease comprising inoculating a mammal with a nucleic acid immunogen comprising a gene, or portion thereof, encoding a cancer or disease-associated antigen, or immunodominant epitope thereof, in combination with an exogenous cytokine adjuvant, and optionally, providing an exogenous immunostimulatory molecule in combination with the immunogen and cytokine adjuvant, wherein the nucleic acid immunogen is directly delivered to the mammal by particle-mediated gene delivery.

47. The method according to claim 46, wherein the cancer is selected from the group consisting of non-Hodgkins lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, carcinoma,

neuroblastoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

48. The method according to claim 46, wherein the disease is caused by a pathogenic microorganism selected from the group consisting of viruses, bacteria, protozoans, parasites, and yeast.

49. The method according to claim 46, wherein the nucleic acid immunogen is affixed to gold particles.

50. The method according to claim 46 or claim 49, wherein the nucleic acid is delivered by means of a hand-held gene gun device.

51. The method according to claim 50, wherein the nucleic acid is delivered into the abdominal epidermis.

52. The method according to claim 46, wherein the exogenous cytokine or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, RANTES, B7.1, B7.2, IL-1, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

53. The method according to claim 52, wherein the exogenous cytokine is selected from the group consisting of IL-2, IL-6, IL-7, and IL-12.

54. A method of making an immune response-enhancing immunogen specifically directed against a disease causing agent or cancer, comprising affixing to particles an isolated nucleic acid sequence encoding one or more antigens or immunodominant epitopes of the disease-causing agent or cancer

and an isolated nucleic acid sequence encoding one or more cytokines, and optionally, an isolated nucleic acid sequence encoding one or more immunostimulatory molecules, wherein the coated particles are directly inoculated into a mammal by means of a direct gene delivery device.

55. An immune response-enhancing nucleic acid immunogen made according to the method of claim 54.

56. A method of preventing a disease or cancer in a mammal, comprising administering to the mammal an effective amount of the pharmaceutical composition according to claim 42, said amount effective to result in the induction of antigen-specific cellular and humoral immune reponses and protective immunity in the mammal, thereby resulting in the reduction of the disease or cancer.

57. A method of preventing a disease or cancer in a mammal, comprising administering to the mammal an effective amount of the pharmaceutical composition according to claim 44, said amount effective to result in the induction of antigen-specific cellular and humoral immune reponses and protective immunity in the mammal, thereby resulting in the reduction of the disease or cancer.

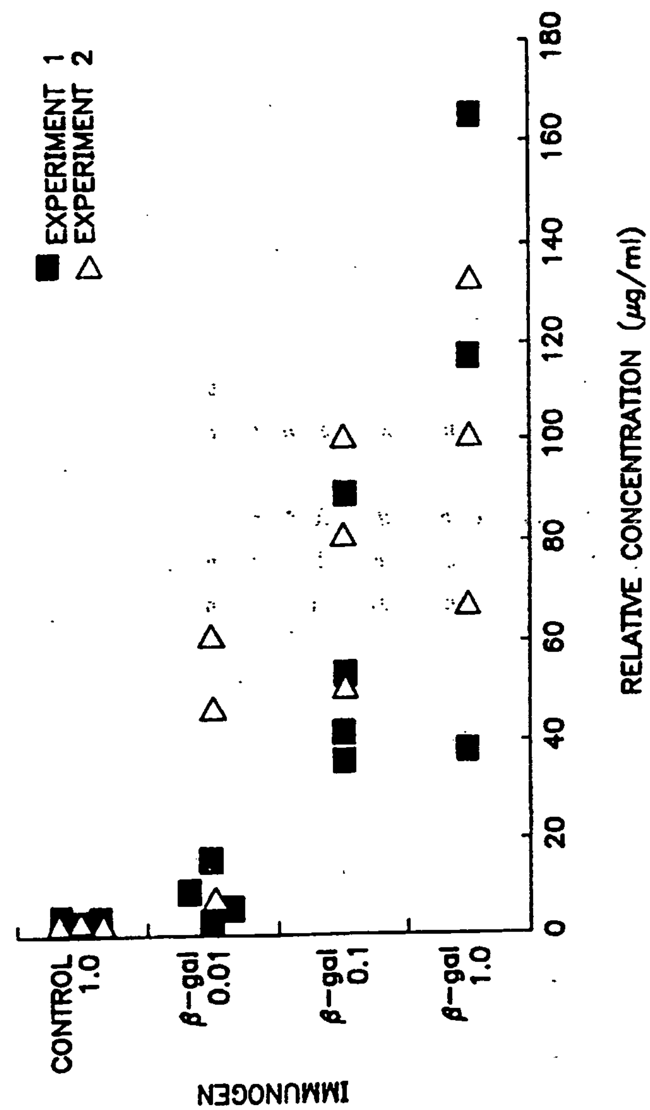
EXPERIMENT 1
EXPERIMENT 2
CONTROL 1.0
β−gal 0.01
β−gal 0.1
β−gal 1.0
IMMUNOGEN
0
20
40
60
80
100
120
140
160
180
RELATIVE CONCENTRATION (μg/ml)

FIG. I

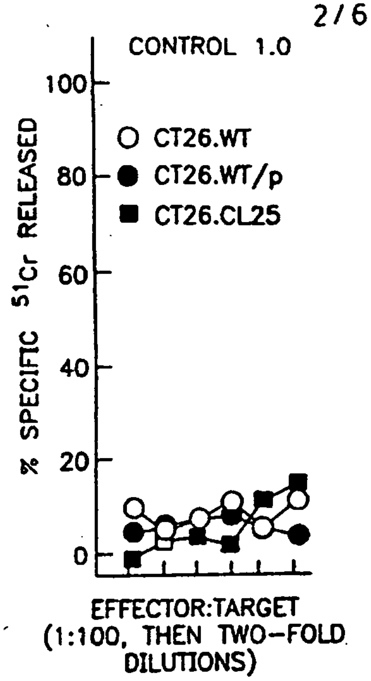

FIG. 2A

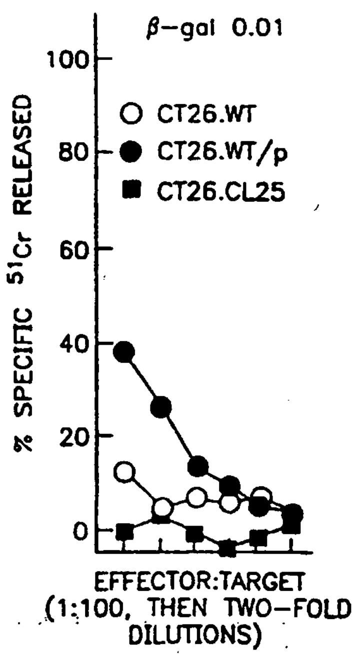

FIG. 2B

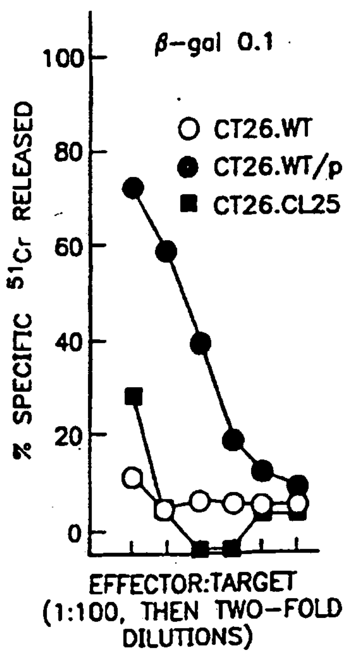

FIG. 2C

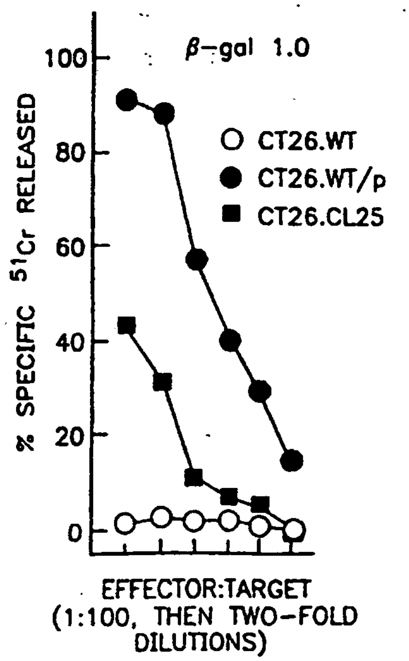

FIG. 2D

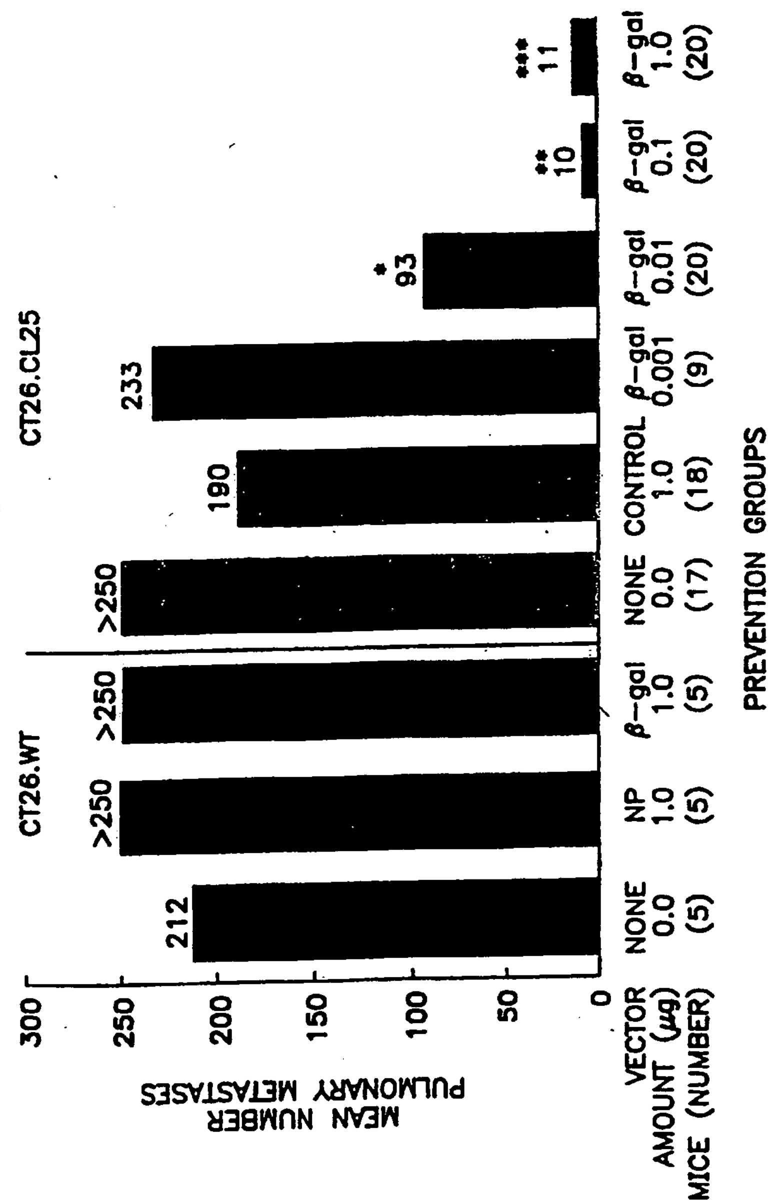
CT26.WT
CT26.CL25
MEAN NUMBER PULMONARY METASTASES
300
250
200
150
100
50
0
212
>250
>250
>250
190
233
* 93
** 10
*** 11
VECTOR AMOUNT (μg) MICE (NUMBER)
NONE 0.0 (5)
NP 1.0 (5)
β−gal 1.0 (5)
NONE 0.0 (17)
CONTROL 1.0 (18)
β−gal 0.001 (9)
β−gal 0.01 (20)
β−gal 0.1 (20)
β−gal 1.0 (20)
PREVENTION GROUPS

FIG. 3

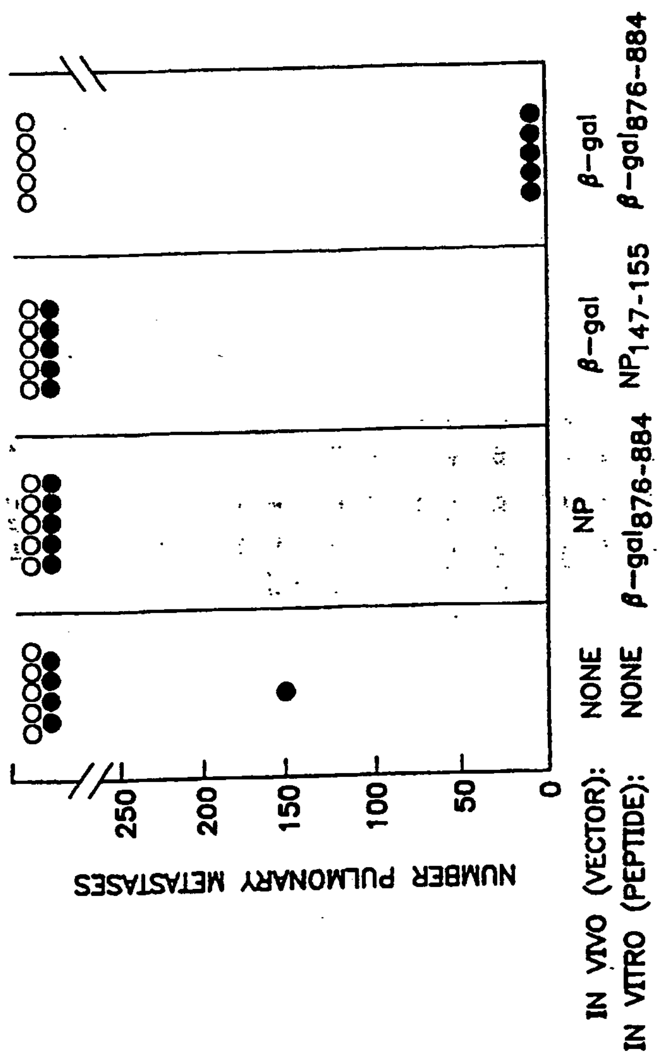
NUMBER PULMONARY METASTASES
250
200
150
100
50
0
IN VIVO (VECTOR): NONE NP β−gal β−gal
IN VITRO (PEPTIDE): NONE β−gal876-884 NP147-155 β−gal876-884
TREATMENT GROUPS

FIG. 4

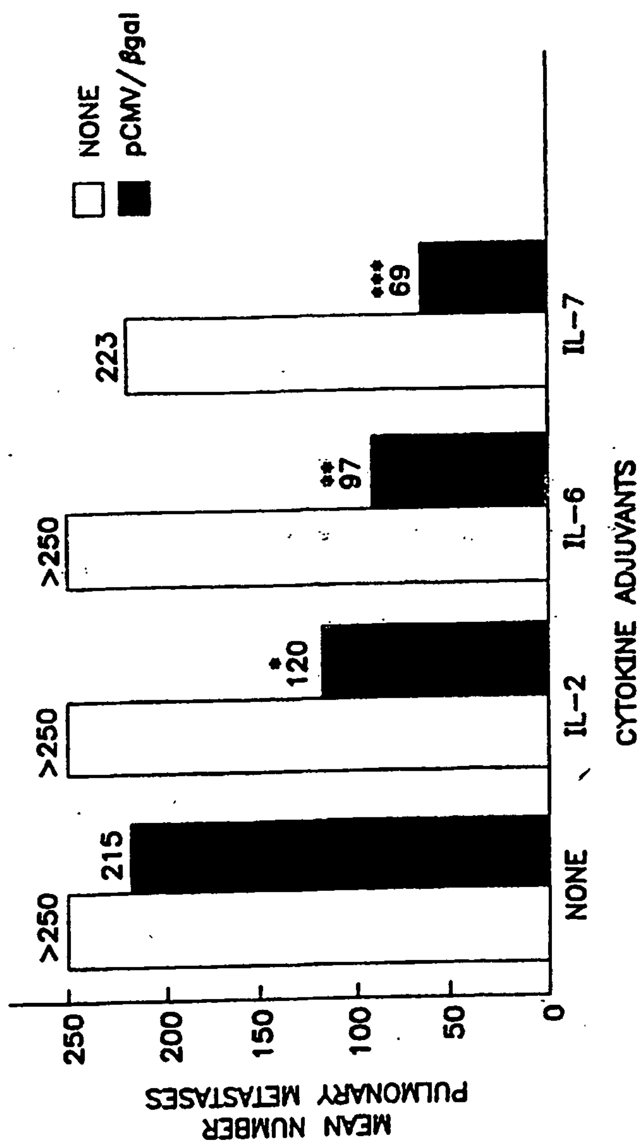
NONE
pCMV/βgal
>250
215
>250
* 120
>250
** 97
223
*** 69
NONE
IL-2
IL-6
IL-7
CYTOKINE ADJUVANTS
MEAN NUMBER PULMONARY METASTASES
250
200
150
100
50
0

FIG. 5

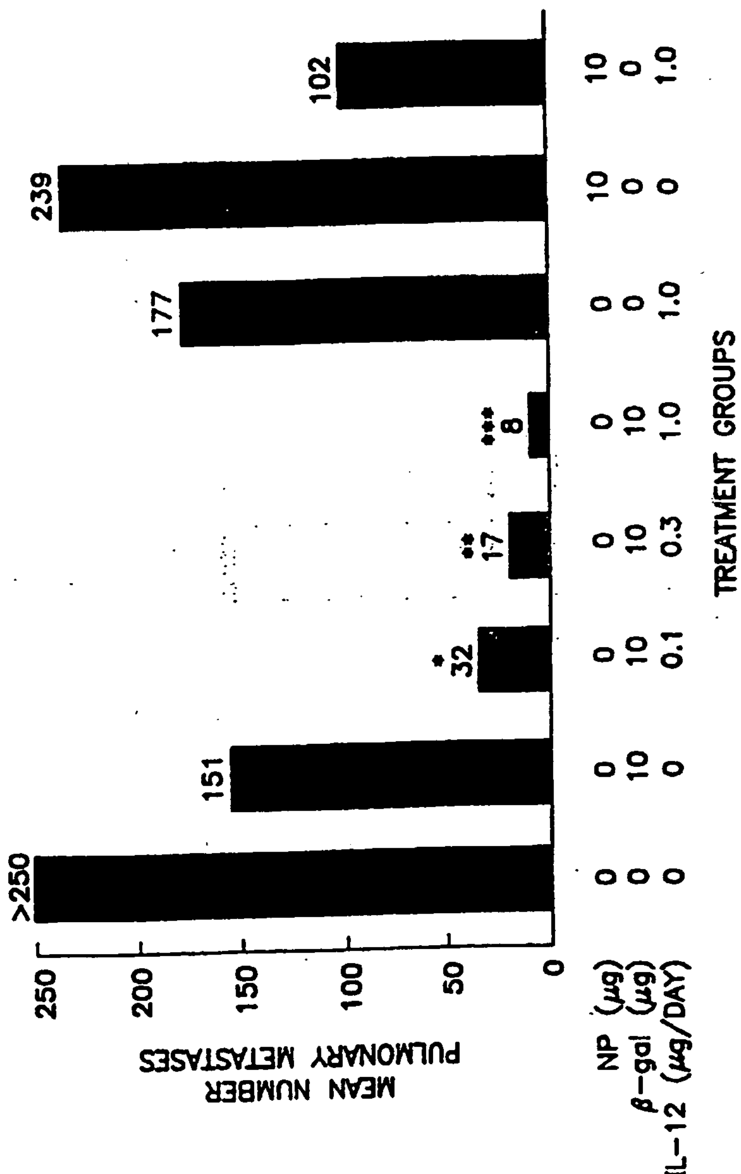
MEAN NUMBER
PULMONARY METASTASES
250
200
150
100
50
0
>250
151
32
17
8
177
239
102
*
**
***
NP (μg)
β−gal (μg)
IL−12 (μg/DAY)
0 0 0
0 10 0
0 10 0.1
0 10 0.3
0 10 1.0
0 0 1.0
10 0 0
10 0 1.0
TREATMENT GROUPS

FIG. 6

INTERNATIONAL SEARCH REPORT

Int : Application No
PCT/US 96/20571

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/24 C12N15/25 C12N15/26 A61K39/00
A61K39/002 A61K39/02 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGICAL REVIEWS, no. 145, June 1995, pages 211-228, XP000670867 STEVENSON ET AL: "IDIOTYPIC DNA VACCINES AGAINST B-CELL LYMPHOMA"	1-5, 7-16, 18-57
Y	see the whole document ---	6,17
X	IMMUNITY, vol. 2, February 1995, pages 129-135, XP000670098 XIANG ET AL: "MANIPULATION OF THE IMMUNE RESPONSE TO A PLASMID-ENCODED VIRAL ANTIGEN BY COINOCULATION WITH PLASMIDS EXPRESSING CYTOKINES"	1-5,7-9, 11-16, 18, 20-32, 34-40, 42-52, 54-57
Y	see the whole document ---	6,17

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
7 May 1997	20.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 96/20571

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05853 A (UNIV CALIFORNIA ;CARSON DENNIS A (US); RAZ EYAL (US); HOWELL MERED) 2 March 1995 see page 14, line 14 - page 22, line 20 ---	1-3, 9-16, 18-26, 29-34, 37-46, 49-57
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT 21A, 10 March 1995 - 4 April 1995, page 175 XP002030629 RALSTON ET AL: "COMPARISON OF CELLULAR IMMUNITY TO MAGE TUMOR ANTIGENS ELICITED IN MICE USING PEPTIDES, PURIFIED PROTEIN, VACCINIA VIRUS, AND POLYNUCLEOTIDE VACCINES" see abstract C2-565 ---	6,17
Y	WO 95 29193 A (US HEALTH) 2 November 1995 see page 4, line 14 - page 6, line 21 ---	6,17
Y	THE JOURNAL OF IMMUNOLOGY, vol. 154, 15 May 1995, pages 5282-5292, XP002030630 BRONTE ET AL: "IL-2 ENHANCES THE FUNCTION OF RECOMBINANT POXVIRUS-BASED VACCINES IN THE TREATMENT OF ESTABLISHED PULMONARY METASTASES" cited in the application see page 5282, summary ---	6,17
Y	VACCINES 96. MOLECULAR APPROACHES TO THE CONTROL OF INFECTIOUS DISEASES, 1995, pages 45-49, XP000673326 TASCON ET AL: "POLYNUCLEOTIDE VACCINATION INDUCES A SIGNIFICANT PROTECTIVE IMMUNE RESPONSE AGAINST MYCOBACTERIA" see the whole document ---	1-57
P,X	WO 96 13277 A (UNIV CALIFORNIA) 9 May 1996 see page 15, line 14 - page 19, line 15 ---	1-3, 9-16, 18-26, 29-34, 37-46, 49-57
P,Y	WO 96 11279 A (US HEALTH) 18 April 1996 see page 18, line 20 - page 21, line 30 ---	1-57

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 96/20571

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 156, 1 January 1996, pages 238-245, XP002030631 IRVINE ET AL: "CYTOKINE ENHANCEMENT OF DNA IMMUNIZATION LEADS TO EFFECTIVE TREATMENT OF ESTABLISHED PULMONARY METASTASES" see the whole document -----	1-57

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/20571

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 24-41,46-53,56,57
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 24-41,46-53,56,57 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No
PCT/US 96/20571

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505853 A	02-03-95	AU 7639194 A	21-03-95
		EP 0714308 A	05-06-96
		JP 9501936 T	25-02-97
WO 9529193 A	02-11-95	AU 2395895 A	16-11-95
		CA 2188432 A	02-11-95
		EP 0756604 A	05-02-97
		FI 964235 A	20-12-96
WO 9613277 A	09-05-96	AU 4199496 A	23-05-96
WO 9611279 A	18-04-96	AU 3998295 A	02-05-96